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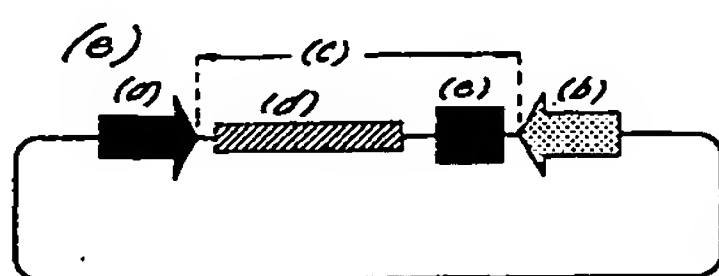
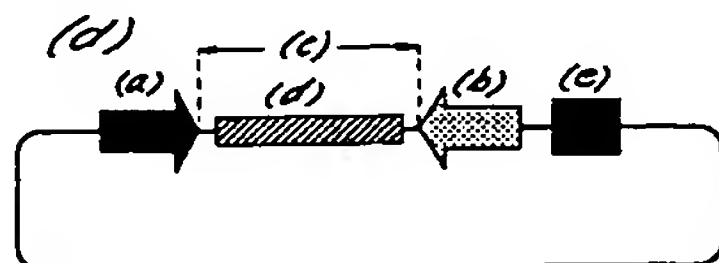
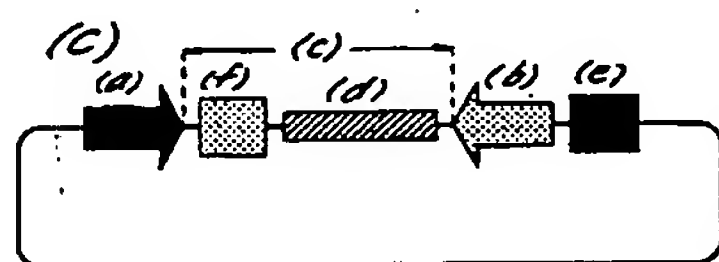
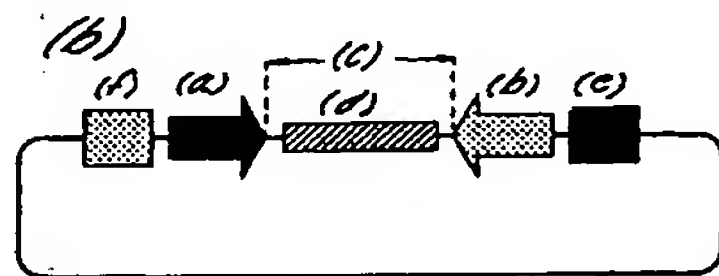
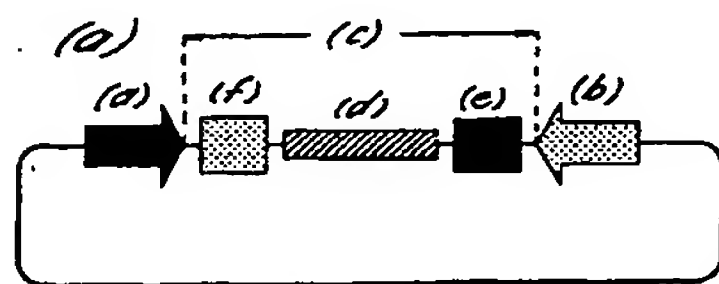
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(54) Title: **VECTOR CONSTRUCTS**



(a): promoter 1  
(b): promoter 2  
(c): Terminator 1  
(d): Terminator 2

(57) Abstract: Vector constructs useful in the expression of double-stranded RNA. The constructs are particularly useful for expression of double-stranded RNA in vitro and in vivo.

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## VECTOR CONSTRUCTS

### Field of the invention

The invention relates to improved vector  
5 constructs for use in the expression of double-  
stranded RNA, particularly for use in the expression  
of double-stranded RNA *in vitro* and *in vivo*.

### Background to the invention

10 Since the advent of double-stranded RNA  
inhibition (RNAi) as a tool for controlling gene  
expression, as described in WO 99/32619 and WO  
00/01846, there has been recognised a need for  
specialised vectors designed for the production of  
15 double-stranded RNA (dsRNA).

Cloning vectors designed to produce high levels  
of dsRNA have been previously described by Plaetinck  
et al. (WO 00/01846) and Timmons et al. Nature,  
395:854 (1998). These vectors generally contain a  
20 multiple cloning site (MCS) into which target DNA  
fragments can be cloned flanked by two opposable  
transcriptional promoters. Essentially, these three  
components (Promoter 1, MCS and Promoter 2) make up  
the entire system. In the appropriate expression  
25 system, the DNA cloned into the MCS may be transcribed  
in both directions, leading to the production of two  
complementary RNA strands.

A disadvantage of the known systems is that not  
only the cloned fragment is transcribed. Read-through  
30 of the RNA polymerase will result in transcription of  
the entire vector, and this also in both directions.  
As only transcription of the cloned DNA fragment will  
result in active dsRNA for RNAi purposes,  
transcription of the vector part results in useless,

inefficient RNA. More specifically, 80% of these transcripts can be considered as non-specific and thus non-effective.

The large amounts of non-specific RNA generated by the prior art plasmid and expression systems results in some undesirable side effects. First, in RNAi protocols based on introduction of dsRNA into *C. elegans* via a food organism such as *E. coli* which expresses the dsRNA (see WO 00/01846), large RNA strands are considered to be toxic for the food organism. As a result, high amounts of RNA accumulating in *E. coli* cause a significant part of the population to die. Second, and probably more important, is the reduction of inhibition potential. The presence of large amounts of non-specific dsRNA causes a competitive environment for the specified sequences. The potential of the template-specified dsRNA sequences to inhibit the targeted protein expression in, for instance, *C. elegans* cells is reduced by the presence of these large non-specific regions. Such an inhibition by non-specific dsRNA has also been shown in *Drosophila* by Tushl et al., Genes & Development 13:3191-3197 (1999). Not only the potential to inhibit gene expression is affected, but also the amount of specific dsRNA produced is limited. Third, transcription of the vector backbone part, more particularly transcription of the origin of replication and related structures, results in plasmid instability and plasmid reorganisation, leading to reduced production of dsRNA. This relatively low concentration of effective dsRNA in turn leads to inefficient RNAi.

To conclude, the previously described vectors have following shortcomings: they are toxic to the

feeding organism, a greater proportion of the transcripts produced are non-specific, the inhibitory potential of the dsRNA is reduced by the presence of non-specific regions, a high incidence of plasmid reorganizations and loss of plasmid from the feeding organism. It is therefore an object of the present invention to provide improved vectors for the production of dsRNA which avoid the disadvantages of the prior art vectors.

10 Vectors for use in the *in vitro* synthesis of RNA transcripts, for example the production of RNA probes, have been known and commonly used in the art for some time (see for example F. M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994); Jendrisak et al, Vectors for in vitro production of RNA copies of either strand of a cloned DNA sequence, US 4,766,072). In standard in vitro transcription protocols the problem of read-through transcription of vector sequences is generally avoided by linearizing the transcription vector at restriction site positioned at the 3' end of the desired transcript. However, this solution is not appropriate for *in vivo* transcription or for the production of dsRNA where it is important that the template is transcribed in both directions.

25 The inventors now propose a novel solution to the problems encountered with the prior art vectors for the production of dsRNA, based on the use of transcription terminators. Generally the solution consists of the use of at least one transcription terminator operably linked to at least one promoter, wherein the terminator stops the transcription initiated by the promoter. Any DNA fragment inserted between the 3' end of the promoter and the 5' end of

the terminator will then be transcribed, without the unwanted transcription of the vector backbone.

Preferentially the vector consists of two promoters and two terminators, as further described below.

5       Therefore, in accordance with a first aspect of the invention there is provided a DNA construct comprising two opposable promoters flanking an inter-promoter region, the construct further comprising at least one transcription terminator positioned  
10 transcriptionally downstream of one of the said promoters In particular, the invention provides for:  
a DNA construct comprising:

- a) a first promoter and
- b) a second promoter,

15 in which the first and second promoter are in opposite orientation to each other and define:

- c) an inter-promoter region positioned downstream of the 3' end of the first promoter and downstream of the 3' end of the second promoter;

20 and which DNA construct further comprises:

- d) at least one cloning site positioned in the inter-promoter region; and

e) a first transcription terminator, positioned (as seen from the 3' end of the first promoter)  
25 downstream of the first promoter and downstream of the at least one cloning site, wherein the first transcription terminator is operably linked to the first promoter.

The inter-promoter region can also further be  
30 defined as: the DNA region between the 3' end of the first promoter and the 3' end of the second promoter, and which is downstream of the first promoter, and which is downstream of the second promoter, and which preferably does not contains the 5' end of the first



- 5 -

promoter and of the second promoter. The opposable first promoter and second promoter drive expression directional from their 5' ends to their 3' ends starting transcription downstream of their 3' ends, thus providing transcription of both strands of any nucleotide sequence(s) present in the inter-promoter region.

The two promoters present in the DNA construct of the invention may be identical or they may be different and may be of essentially any type. The precise nature of the promoters used in the construct may be dependent on the nature of the expression system in which the construct is expected to function (e.g. prokaryotic vs eukaryotic host cell).

Bacteriophage promoters, for example the T7, T3 and SP6 promoters, are preferred for use in the constructs of the invention, since they provide advantages of high level transcription which is dependent only on binding of the appropriate RNA polymerase. Each of these promoters can independently be chosen. The phage promoters can also function in a wide variety of host systems, i.e. both prokaryotic and eukaryotic hosts, provided that the cognate polymerase is present in the host cell.

The arrangement of two "opposable" promoters flanking an inter-promoter region such that transcription initiation driven by one of the promoters results in transcription of the sense strand of the inter-promoter region and transcription initiation driven by the other promoter results in transcription of the antisense strand of the inter-promoter region is an arrangement well known in the art, for example, in the pGEM7 series of vectors from Promega Corp., Madison WI, USA.

The DNA constructs of the invention differ from those of the prior art because of the presence of at least one transcription terminator positioned transcriptionally downstream of one of the promoters.

5 The transcription terminator may be uni- or bi-directional, the choice of uni- vs bi-directional terminators being influenced by the positioning of the terminator(s) within or outside the inter-promoter region, as explained below. The terminator may be of  
10 prokaryotic, eukaryotic or phage origin. Bacteriophage terminators, for example T7, T3 and SP6 terminators, are particularly preferred. The only requirement is that the terminator must be capable of causing termination of transcription initiating at the  
15 promoter relative to which it is transcriptionally downstream. In practice, these means that the promoter and terminator must form a 'functional combination', i.e. the terminator must be functional for the type of RNA polymerase initiating at the  
20 promoter. By way of example, a eukaryotic RNA pol II promoter and a eukaryotic RNA pol II terminator would generally form a functional combination. The selection of a functional combination is particularly important where bacteriophage promoters and  
25 terminators are to be used in the constructs of the invention, since the phage promoters and terminators are both polymerase-specific. To form a functional combination both the promoter and the terminator should be specific for the same polymerase, e.g. T7  
30 promoter and T7 terminator, T3 promoter and T3 terminator etc.

In one embodiment, the DNA construct of the invention may comprise a single transcription terminator, positioned (as seen from the 3' end of the



first promoter) downstream of the first promoter and downstream of the at least one cloning site, wherein the first transcription terminator is operably linked to the first promoter, wherein the single

5 transcription terminator is positioned in the inter-promoter region

In an alternative arrangement, the DNA construct comprises a single transcription terminator positioned outside of the inter-promoter region. In a still  
10 further embodiment, the DNA construct may comprise two transcription terminators, each one of which is positioned transcriptionally downstream of one of the two promoters. In this arrangement, one or both of the terminators may be positioned within the inter-  
15 promoter region. These various embodiments of the DNA constructs of the invention will be more fully described below, with reference to the accompanying drawings. The position of a first transcription terminator outside the inter-promoter region may also  
20 be further defined as, i.e. such that a first transcription terminator is positioned (as seen from the 3' end of the first promoter) downstream of the first promoter, downstream of the at least one cloning site, and downstream of the 5' end of the second  
25 promoter.

The position of a second transcription terminator outside the inter-promoter region may also be further defined as, i.e. such that a second transcription terminator positioned (as seen from the 3' end of the  
30 second promoter) downstream of the second promoter, downstream of the at least one cloning site, and downstream of the 5' end of the first promoter.

Moreover, when the terminator is not located in the inter-promoter region, the distance between the 5'

end of the first promoter and 3' end of the second terminator, or the distance between the 5' end of the second promoter and the 3' end of the first terminator is preferably small, i.e. such that the 3' end of the first transcription terminator is separated from the 5' end of the second promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferable no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10 nucleotides, more particularly preferably no more than 6 nucleotides.

Furthermore, when the second transcription terminator is located outside of the inter-promoter region, preferably the 3' end of the second transcription terminator is separated from the 5' end of the first promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10 nucleotides, more particularly preferably no more than 6 nucleotides

As defined above the term 'inter-promoter region' refers to all of the DNA sequence between the two promoters. As explained above, in certain embodiments of the invention the transcription terminator(s) may be sited within the inter-promoter region. The inter-

- 9 -

promoter region may, advantageously, comprise a sequence of nucleotides forming a template for dsRNA production. The precise length and nature of this sequence is not material to the invention. The invention further provides DNA constructs in which the inter-promoter region comprises a cloning site. The function of the cloning site is to facilitate insertion of a DNA fragment forming a template for dsRNA production between the two promoters. Thus, the invention provides a series of cloning vectors which are of general use in the construction of template vectors for dsRNA production. Also encompassed within the scope of the invention are vectors derived from the cloning vectors which have a DNA fragment inserted into the cloning site.

The cloning site may further comprise one or more of the following:

- at least one restriction site, (as known in the art), or one or more further restriction sites, e.g. to provide a multiple cloning site (as known in the art),
- a stuffer DNA, e.g., flanked by at least two restriction site, such as two *Bst*XI restriction sites, or two *Xcm*I restriction sites,
- *att*R1 and *att*R2 recombination sites,
- a *ccdB* nucleotide sequence,
- a *ccdB* nucleotide further comprising at least one unique blunt-end restriction site, such as a *Srf*I restriction site, and/or

- a DNA fragment inserted in the at least one cloning site. All of the DNA constructs provided by the invention may, advantageously, form part of a replicable cloning vector, such as, for example, a plasmid vector. In addition to the opposable

- 10 -

promoters, inter-promoter region and transcription terminator(s), the vector 'backbone' may further contain one or more of the general features commonly found in replicable vectors, for example an origin of replication to allow autonomous replication within a host cell and a selective marker, such as an antibiotic resistance gene. The selective marker gene (e.g. the antibiotic resistance gene) may itself contain a promoter and a transcription terminator and it is to be understood that these are completely independent of the promoter and terminator elements required by the invention and are not to be taken into consideration in determining whether a particular vector falls within the scope of the invention.

DNA constructs according to the invention may be easily be constructed from the component sequence elements using standard recombinant techniques well known in the art and described, for example, in F. M. Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994), as will be appreciated by one skilled in the art from the following detailed description of the invention and the accompanying Examples.

There follows a detailed description of DNA constructs according to the invention, with reference to the following schematic drawings in which:

Figures 1(a) to 1(e) are schematic representations of several different embodiments of the DNA construct according to the invention illustrating the relative positioning of the promoter and transcription terminator elements.

Figure 2(a) is a schematic representation of a prior

- 11 -

art vector included for comparison purposes.

Figures 2(b) to 2(e) are schematic representations of several further embodiments of the DNA construct  
5 according to the invention illustrating the use of different cloning sites in the inter-promoter region.

Referring to the Drawings, Figure 1(a) schematically illustrates a first DNA construct  
10 according to the invention which is a plasmid vector comprising two opposable promoters; a first promoter a) and second promoter b) flanking an inter-promoter region c), which inter-promoter region is downstream of the 3' of the first promoter, and down  
15 stream of the 3' end of the second promoter. The first promoter and the second promoter may be identical or different. This embodiment comprises a first transcription terminators e) and a second transcription terminator f) both of which are  
20 positioned within the inter-promoter region. In this embodiment, the first terminator and the second terminator are preferentially uni-directional terminators.

A DNA fragment may be inserted in the at least  
25 one cloning site d). Such fragment is subject to transcription directed by the first promoter a) and the second promoter b) (i.e. transcription of both strands), resulting in the generation of two RNA fragments which may combine to double-stranded RNA of  
30 the inserted DNA fragment (both *in vitro* and *in vivo*).

Any desired DNA sequence, such as a genomic DNA sequence, or a cDNA sequence or any other coding sequence, may be inserted in the at least one cloning site. Without being limited to any specific

- 12 -

explanation, it is assumed that when a) and e) form a functional combination, RNA polymerase which initiates transcription at a) will transcribe the inter-promoter region including the at least cloning site and the DNA fragment inserted in the at least cloning site and will be terminated when it reaches e). Similarly, RNA polymerase which initiates transcription at b) will transcribe the inter-promoter region including the at least one cloning site and the DNA fragment inserted in the at least one cloning site and will terminate when it reaches f). The terminators cause the RNA polymerase to pause, stop transcription and fall off the template. This prevents the unlimited transcription of the vector backbone, and reduces the unspecific transcription of non-essential DNA.

The inter-promoter region further comprises a sequence of nucleotides corresponding to a target for double-stranded RNA inhibition. This sequence is designated 'TF' for target fragment. It is this sequence which, when transcribed into dsRNA, will be responsible for specific double-stranded RNA inhibition of a target gene. The target fragment may be formed from a fragment of genomic DNA or cDNA from the target gene. Its precise length and nucleotide sequence are not material to the invention.

In the arrangement shown in Figure 1(a) the two terminators are positioned on either side of the TF within the inter-promoter region. Each of the terminators is positioned transcriptionally downstream of one of the promoters, the first terminator e) is transcriptionally downstream of first promoter a) and the second terminator f) is transcriptionally downstream of the second promoter b). Assuming that a) and e) form a functional combination, as described



- 13 -

above, RNA polymerase which initiates transcription at  
a) will transcribe the inter-promoter region up to and  
including TF and will be terminated when it reaches  
e). Similarly, RNA polymerase which initiates  
5 transcription at b) will transcribe the inter-promoter  
region up to and including TF on the opposite strand  
and will terminate when it reaches f). The  
terminators cause the RNA polymerase to pause, stop  
transcription and fall off the template. This  
10 prevents the unlimited transcription of the vector  
backbone, and reduces the unspecific transcription of  
non-essential DNA.

The transcripts generated from this vector may,  
depending on the precise placement of the terminators  
15 in the vector, be almost completely specific dsRNAs  
corresponding to the TF region. Through the direct  
placement of the terminator sequences at the  
downstream end of the TF region on both sides of the  
inserted DNA fragment, the amount of material  
20 transcribed is completely reduced to the  
template-specified sequences. Therefore, a higher  
amount of specific dsRNA is obtained. Furthermore the  
constructs are now also more stable, due to the  
non-transcription of the vector backbone. The latter  
25 characteristic (stability), combined with the now  
relatively higher specific transcription rate,  
provides a system adapted to synthesise higher amounts  
of specific short dsRNA strands. This proportionally  
higher amount of transcript, resulting in high  
30 concentrations of specific dsRNA, enhances the  
inhibitory effect in RNAi protocols. In RNAi  
protocols based on expression of dsRNA in a food  
organism, toxicity for the feeding organisms due to  
high RNA expression is brought to a minimal level by

use of this vector.

A specific example of a vector of the type illustrated in Figure 1(a), considered by the inventors to be the optimal arrangement for RNAi applications, is plasmid pGN9 described in the accompanying Examples. The transcriptional terminators used in pGN9 are T7 RNA polymerase specific terminators, since the vector contains two opposable T7 promoters. However, other systems could be used such as an expression system based on the T3 or SP6 promoter, terminator and polymerase or other prokaryotic or eukaryotic promoters and terminators.

Figure 1(b) illustrates schematically a further DNA construct according to the invention which is a plasmid vector comprising two opposable promoters a) and b) flanking an inter-promoter region c). This vector also comprises two transcription terminators e) and f) but in this arrangement the two terminators are positioned outside of the inter-promoter region, in fact the terminator elements now flank the two promoters. The arrangement is such that e) is transcriptionally downstream of a) whilst f) is transcriptionally downstream of b). Here again e) terminates the transcription initiated by a), whilst f) terminates the transcription initiated by promoter b). Placement of the terminators outside of d) allows the use of bi-directional terminators as well as uni-directional terminators, in contrast to the arrangement in Figure 1(a) where uni-directional terminators are preferred because of the placement of the terminators between the promoters. A number of bi-directional terminators which could be used in accordance with the invention are known in the art.

- 15 -

Generally these are observed to be polymerase non-specific.

The embodiment shown in Figure 1(b) provides essentially the same advantages as that shown in Figure 1(a) over the prior art vectors for dsRNA production. The vector shown in Figure 1(b) will lead to the production of transcripts which are slightly longer, including the promoter regions. This relatively small difference in the length of the transcript and hence the formed dsRNA will not severely affect the efficacy in an RNAi system.

The position of the terminators and promoter in the example as shown in figure 1 (b) are preferably placed at close proximity, such that the 5' end of the promoters are separated from the 3' end of the transcription terminators by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10 nucleotides, more particularly preferably no more than 6 nucleotides.

Figure 1(c) illustrates schematically a further DNA construct according to the invention which is a plasmid vector comprising two opposable promoters a) and b) flanking an inter-promoter region c). In this embodiment one terminator (in this case e)) is positioned within the c) and the other (f)) is positioned outside c). Again, e) terminates transcription initiated by a) and f) terminates

- 16 -

transcription initiated by b). This arrangement may provide a useful solution to the problem of one of the terminators interfering with polymerase activity in the other direction (e.g. f) interferes with b) initiated transcription). This vector essentially provides the same advantages as the vector variations shown in Figure 1(a) and Figure 1(b) over the prior art. The relatively small difference in the length of the transcript due to the transcription of one of the promoters will not significantly affect the efficacy in RNAi systems. This more particularly the case when the terminator that is located outside of the inter-promoter region c) is at close proximity of the promoter, as defined above.

Figures 1(d) and 1(e) illustrate schematically two further DNA constructs according to the invention which are both plasmid vectors comprising two opposable promoters a) and b) flanking an inter-promoter region c). These embodiments comprise a single terminator only. In the arrangement shown in Figure 1(d) a single terminator e) which terminates transcription from a) is placed outside of c). The placement of the terminator outside of the IPR allows the use of both a bi-directional terminator or a uni-directional terminator in this system. In the embodiment shown in Figure 1(d) e) is placed within the c). a) should therefore preferably be a uni-directional terminator.

Further embodiments of the DNA construct according to the invention are illustrated schematically in Figures 2(b) to 2(e).

These embodiments are all plasmid cloning vectors, based upon the optimal arrangement of promoters and terminators shown in Figure 1(a), and

- 17 -

described above, containing cloning sites to facilitate the insertion of a DNA fragment into the at least on cloning site.

These embodiments are all plasmid cloning  
5 vectors, based upon the optimal arrangement of promoters and terminators shown in Figure 1(a), containing cloning sites to facilitate the insertion of a target DNA fragment into the inter-promoter region.

10 Figure 2(a), which is a schematic representation of a prior art cloning vector, is included for comparison purposes. This vector comprises two opposable promoters a) and b), which may be identical or different, flanking a multi-cloning site (MCS).

15 Figure 2(b) illustrates a first type of plasmid cloning vector according to the invention. The vector contains a first opposable promoters a) and a second opposable promoter b) flanking an inter-promoter region. The inter-promoter region can further be  
20 defined as: the DNA region between the 3' end of the first promoter and the 3' end of the second promoter, and which is downstream of the first promoter, and which is downstream of the second promoter, and which preferably does not contains the 5' end of the first  
25 promoter and of the second promoter. The inter-promoter promoter region further comprises terminators e) and f) flanking a multi-cloning site MCS. The MCS comprises at least one individual restriction sites, an preferably more than one  
30 restriction site as known in the art, any of which may be used for insertion of a DNA fragment.

Figure 2(c) illustrates a further type of plasmid cloning vector according to the invention. This vector again contains opposable promoters a) and b)

flanking an inter-promoter region comprising terminators e) and f). In this embodiment, a) and b) flank a cloning site which is adapted for facilitated cloning of PCR fragments, comprising a stuffer DNA  
5 flanked by two identical restriction sites, in this case BstXI sites. The specific sequence of the stuffer DNA is not essential, provided that said stuffer DNA does not interfere with the desired effect and/or the desired activity of the DNA constructs of the  
10 invention. A specific example of a vector according to this aspect of the invention described herein is plasmid pGN29.

The cloning of PCR products using BstXI recognition sites and BstXI adaptors is generally  
15 known in the art. The BstXI adaptors are commercially obtained, such as from Invitrogen (Groningen, the Netherlands). These adaptors are non-palindromic adapters designed for easier and efficient cloning of PCR products into vectors. The use of these adaptors  
20 reduces the concatemerization of adapters or insert DNA by having non-complementary (CACA) overhangs. The stuffer DNA is included merely to allow easy differentiation between BstXI cut and uncut vector on the basis of size. Its precise length and sequence  
25 are not of importance.

Figure 2(d) illustrates a further type of plasmid cloning vector according to the invention. This vector again contains opposable promoters a) and b) flanking an inter-promoter region comprising  
30 terminators e) and f). In this embodiment, a) and b) flank a cloning site which facilitates "High Throughput" cloning based on homologous recombination rather than restriction enzyme digestion and ligation. As shown schematically in Figure 2(d), the cloning



- 19 -

site comprises attR1 and attR2 recombination sites from bacteriophage lambda flanking a gene which is lethal to *E. coli*, in this case the *ccdB* gene.

5 An alternative cloning method of DNA fragments into this vector, (not shown in Figure 2 (d)), consists of a variant of this vector, wherein the *ccdB* DNA sequence further comprises at least one unique restriction site, preferably the at least unique restriction site is a blunt-end restriction site, such  
10 as a *SrfI* restriction site. Insertion of a DNA fragment in the at least unique restriction, results in inactivation of the *ccdB* gene, and hence in inactivation of the lethal *ccdB* gene.

A further variant of a vector is shown in Figure  
15 2(d) in which the attR1 and the attE2 are not present. Such a vector comprises an at least one cloning site, said at least one cloning site consisting of a *ccdB* sequence, said *ccdB* sequence further comprising at least one unique restriction site, preferably the at  
20 least unique restriction site is a blunt-end restriction site, such as a *SrfI* restriction site. Insertion of a DNA fragment in the at least unique restriction, results in inactivation of the *ccdB* gene, and hence in inactivation of the lethal *ccdB* gene.

25 These cloning sites comprising the *ccdB* nucleotide sequence and/or the attR sites (R1 and/or R2) are derived from the Gateway™ cloning system commercially available from Life Technologies, Inc. The Gateway™ cloning system has been extensively  
30 described by Hartley et al. in WO 96/40724 (PCT/US96/10082). A specific example of a vector according to this aspect of the invention described herein is pGN39.

Figure 2(e) and 2(f) illustrate a still further type of plasmid cloning vector according to the invention. This vector again contains opposable promoters a) and b) flanking an inter-promoter region c) comprising terminators e) and f). In the embodiment shown in Figure 2(e), e) and f) flank a cloning site which facilitates "high throughput" cloning of PCR products by TA<sup>TM</sup> cloning. This cloning site comprises a stuffer DNA flanked by two identical restriction sites for an enzyme which generates overhanging T nucleotides. In this case the restriction sites are XcmI sites, but other sites which are cleaved to generate overhanging T nucleotides could be used with equivalent effect. The overhanging T nucleotides facilitate cloning of PCR products which have a overhanging A nucleotide. This principle is known as TA<sup>TM</sup> cloning. The cut vector with overhanging T nucleotides can be "topomerized" to generate a cloning vector of the type shown schematically in Figure 2(f), by linking the enzyme topoisomerase to the overhanging T nucleotides. The resulting vector also facilitates the cloning of PCR products by the principle known as TOPO<sup>TM</sup> cloning.

Both the TOPO<sup>TM</sup> and TA<sup>TM</sup> cloning systems, although not for the vectors described in this invention, are commercially available from Invitrogen. The TOPO<sup>TM</sup> cloning system has extensively been described by Shuman in WO 96/19497 (PCT/US95/16099). The TA<sup>TM</sup> cloning system has extensively been described by Hernstadt et al. in WO 92/06189 (PCT/US91/07147).

It will be readily appreciated by the skilled reader that whilst Figures 2(b)-2(f) illustrate the inclusion of different cloning sites into a vector of the type illustrated in Figure 1(a), these cloning

- 21 -

sites could be included into any of the DNA constructs of the invention, including those illustrated schematically in Figures 1(b) to 1(e)

5 Application of the DNA constructs of the invention in RNAi technology.

As aforementioned, a major application of the DNA constructs/vectors of the invention is in the production of double stranded RNA for use in RNAi  
10 technology. In particular, the constructs are useful in *in vivo* RNAi protocols in the nematode worm *C. elegans*.

In *C. elegans*, RNAi has traditionally been performed by injection dsRNA into the worm. Fire et  
15 al. describes these methods extensively in International Application No. WO 99/32619. In short, both strands of RNA are produced *in vitro* using commercially available *in vitro* transcription kits. Both strands of RNA are allowed to form dsRNA, after  
20 which the dsRNA is injected into *C. elegans*. The new vector system developed by the present inventors is a drastic improvement on this traditional method. First, the RNA can be produced in one step, for instance by using two identical promoters such as  
25 in the vector pGN9. Second, and more important, due to the presence of terminators the transcripts, and hence the formed dsRNA, will be more specific as only the cloned target fragment will be transcribed. This will result in a more efficient RNAi.

30 A further method to perform RNAi experiments in *C. elegans* has been described by Plaetinck et al. in WO 00/01846. In this method *C. elegans* worms are fed with bacteria which produce dsRNA. The dsRNA passes the gut barrier of the worm and induces the same RNAi

- 22 -

as if the dsRNA has been injected. For these experiments, the preferred *E. coli* strain is HT115 (DE3), and the preferred *C. elegans* strain is nuc-1;gun-1. The improved vectors provided by the invention also improve the efficiency of RNAi in this method, as shown in the example below, as only effective dsRNA is produced.

Another method to perform RNAi has also been described by Plaetinck et al. in WO 00/01846. In short, this method is based on the production of dsRNA in the worm itself. This can be done by using worm promoters in the described vectors, or by using a transgenic worm expressing a polymerase specific for non-*C. elegans* promoters present in the vector, such that this polymerase drives transcription of the dsRNA. The promoters will preferentially be selected from the known bacteriophage RNA promoters, such as T7 or T3 or SP6 RNA promoters, which provide the advantage of a high level of transcription dependent only on the binding of the cognate polymerase.

Plasmid vector DNA can be introduced into the worm by several methods. First, the DNA can be introduced by the traditional injection method (Methods in Cell Biology, Vol 48, *C. elegans* Modern Biological Analysis of an organism, ed. by Epstein and Shakes). Second, the DNA can be introduced by DNA feeding. As has been shown by Plaetinck et al. in WO 00/01846, plasmid DNA can be introduced into the worm by feeding the worm with an *E. coli* strain that harbors the plasmid. Preferentially the *E. coli* strain is OP50, or MC1061 or HT115 (DE3) but any other strain would suit for this purpose. The *C. elegans* strain is preferentially a nuc-1 mutant strain or a nuc-1; gun-1 strain. The plasmid DNA from the *E. coli*

passes the gut barrier and is introduced into the nematode, resulting in the expression of dsRNA. As with the other RNAi methods described above, the use of the new vector system will enhance the RNAi by the production of only specific dsRNA.

The invention will be further understood with reference to the following experimental Examples, together with the following additional Figures in which:

Figure 3 is a representation (plasmid map) of pGN1.

Figure 4 is a representation (plasmid map) of pGN9.

Figure 5 illustrates the nucleotide sequence of a fragment of plasmid pGN1, annotated to show the positions of the opposable T7 promoters.

Figure 6 depicts the nucleotide sequence of the T7 transcription terminator.

Figure 7 illustrates the sequences of oligonucleotides oGN27, oGN28, oGN29 and oGN30 used to insert T7 transcription terminators into pGN1. The positions of the T7 pol terminator sequences and of various restriction sites are marked.

Figure 8 illustrates the nucleotide sequence of a fragment of plasmid pGN9, annotated to show the positions of the opposable T7 promoters and the T7 transcription terminators.

Figure 9 (a) is a representation (plasmid map) of pGN29; (b) is a representation (plasmid map) of pGN39; (c) is a representation (plasmid map) of the plasmid TopoRNAi.

5

Figure 10 shows the complete nucleotide sequence of plasmid pGN9.

10

Figure 11 shows the complete nucleotide sequence of plasmid pGN29.

Figure 12 shows the complete nucleotide sequence of plasmid pGN39.

15

Figure 13 shows the complete nucleotide sequence of plasmid TopoRNAi.

Figure 14 shows the complete sequence of plasmid pGN49A.

20

Figure 15 shows the complete sequence of plasmid pGN59A.

Figure 16 is a representation (plasmid map) of pGN49A.

25

Figure 17 is a representation (plasmid map) of pGN59A.

30



**Example 1-Vector construction.**

5 The starting point for construction of the  
vectors exemplified herein was plasmid pGN1. This  
plasmid, described in the applicant's co-pending  
International Application No. WO 00/01846, contains  
two opposable T7 promoters flanking a multi-cloning  
site.

10 Vector construction was carried out according to  
standard molecular biology techniques known in the art  
and described, for example, in F. M. Ausubel et al.  
(eds.), *Current Protocols in Molecular Biology*, John  
Wiley & Sons, Inc. (1994).

15

**1) Construction of pGN9**

pGN1 was first digested with restriction enzymes  
EcoRI and KpnI. Oligonucleotides oGN27 and oGN28  
(Figure 7) were annealed to generate a double stranded  
20 fragment which was then ligated into the EcoRI/KpnI  
cut vector. The resulting plasmid was re-digested  
with XbaI and HindIII. Oligonucleotides oGN29 and  
oGN30 were annealed to generate a double-stranded  
fragment which was then annealed into the XbaI/HindIII  
25 cut vector. The resulting vector was designated pGN9  
(Figures 4 and 10).

**2) Construction of further cloning vectors**

pGN29 (Figure 9(a); Figure 11) was generated by  
30 replacing the MCS in pGN9 with a stuffer DNA flanked  
by BstXI sites. BstXI adapters are commercially  
available from Invitrogen (Groningen, the  
Netherlands).

pGN39 (Figure 9 (b); Figure 12) generated by following steps; pGN29 was digested with BstXI. BstXI adapters (Invitrogen, Groningen, The Netherlands) were ligated to Cassette A provided by the GATEWAY<sup>TM</sup> system (Life Technologies, Inc.). Cassette A contains attR1, CmR, CcdA, CcdB, attR2. The Cassette A with the adapters were then ligated into the digested pGN29, resulting in pGN39A. pGN39A contains a unique SrfI site in the ccdB gene.

The TopoRNAi vector (figure 9 (c); figure 13) was generated in the following way; pGN29 was digested with BstXI. Using PCR with the primers oGN103 and oGN104 and template pCDM8 (Invitrogen, Groningen, The Netherlands), a stuffer was generated which includes XcmI sites. Onto the PCR product, BstXI adapters were ligated, and the resulting ligation product was ligated in the BstXI digested pGN29 vector resulting in the TopoRNAi vector.

oGN103: 5' TACCAAGGCTAGCATGGTTTATCACTGATAAGTTGG 3'

oGN104: 5' TACCAAGGCTAGCATGGGCCTGCCTGAAGGCTGC 3'

PGN49A was constructed to insert an additional unique non-blunt restriction site and to delete the CmR gene pGN39. Overlap PCR was used. A first PCR was performed with primers oGN126 and oGN127 and PGN39A as template. Using primers oGN128 and oGN129 and the same template a second fragment was generated. Overlap PCR using the resulting fragments and primers oGN126P and oGN129P resulted in a final PCR product. To this final PCR

- 27 -

product, BstXI adapters were ligated, and the ligation product was ligated into pGN29 digested with BstXI. The resulting vector was designated pGN49A.

- 5 A control vector was generated to test the efficiency of the pGN49A cloning vector, such vector should contain the T7 promoters, but not the T7 terminators. For this, the XbaI insert of pGN49A was isolated and cloned in pGN1 digested with the same restriction  
10 enzyme. The resulting vector was designated pGN59A.

oGN126 pGATCTGGATCCGGCTTACTAAAAGCCAGATAACAGTATGC

oGN127 GGAGACTTTATCGCTTAAGAGACGTGCACTGGCCAGGGGGATCACC

oGN128:

- 15 CCAGTGCACGTCTCTTAAGCGATAAAGTCTCCCGTGAACCTTACCCGGTGG

oGN129 pGCTGTGTATAAGGGAGCCTGACATTTATATTCCCCAG

Example 2-To illustrate the usefulness of the improved vectors in RNA.

- 20 This experiment was designed to illustrate the improved efficiency of the improved vectors of this invention in double-stranded RNA inhibition, as compared to the vectors known from the prior art. A significant increase on the efficacy of the system  
25 could be expected, as more effective dsRNA was produced and hence RNAi performed better. The experimental system for this proof of concept experiment was to measure *C. elegans* rescue at 25°C in nuc-1 / pha-1(e2123)ts *C. elegans* mutants by RNAi of  
30 sup35 using dsRNA feeding of pGN-2 (-terminator) and pGN-12 (+ terminator), with PGN-1 (empty vector) as a control and dilutor. The pha-1 ts / sup-35 mutation has extensively been described by Schnabel in WO

99/49066.

The *nuc-1* mutation in *C. elegans* provides for a *C. elegans* strain exhibiting better uptake abilities, such as the uptake of the dsRNA complexes than wild type *C. elegans*. This mutant is deleted in the major DNase enzymes, and inventors have proven in earlier co-pending applications that this *C. elegans* strain results in enhanced RNAi by feeding the nematode with dsRNAs.

The *pha-1(e2123)ts* mutation provides a mutant *C. elegans* strain with a phenotype of survival at 15°C and lethality at 25°C. This lethality is suppressible by the inhibition of *sup-35* expression. RNAi of *sup-35* should thus facilitate the rescue of *pha-1(e2123)ts* at 25°C. The vectors of the present invention, when expressing dsRNA from *sup-35*, should increase the efficacy of *sup-35* RNAi in rescuing *pha-1(e2123)ts* mutants at 25°C, compared to vectors that do not contain the terminators.

Vector pGN1 was used as empty vector. Vector pGN2 (-terminator) is a vector harboring *sup-35* DNA and expressing *sup-35* dsRNA when introduced in the appropriate host, the vector has no transcriptional terminators inserted. Vector pGN12 (+ terminator) is a vector as described above, containing the transcriptional terminators, and hence resulting in improved dsRNA production when introduced into an appropriate host. Thus, this vector has two unidirectional transcriptional terminators, both placed inside of the inter-promoter region, and flanking the *sup-35* fragment. Use of the latter

- 29 -

vector was expected to increase the efficacy of the system, here meaning a better rescue (survival) of pha-1(e2123)ts mutants at 25°C.

#### 5 Experimental conditions

12-well micro-titer plates were filled with approximately 2 ml of NGM agar per well.

(1 liter of NGM agar: 15g Agar, 1g peptone, 3g NaCl, 1ml cholesterol solution (5 mg/ml in EtOH), with  
10 sterile addition after autoclaving of 9.5 ml 0.1M CaCl<sub>2</sub>, 9.5 ml 0.1 ml MgSO<sub>4</sub>, 25 ml 1M KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> buffer pH 6, Ampicillin (100 µg/l), 5ml 0,1M IPTG and 5 ml nystatin solution (dissolved 10 mg/ml in 1:1 EtOH:CH<sub>3</sub>COONH<sub>4</sub> 7.5 M)

15

The dried plates were spotted with approximately 50 µl of an overnight culture of bacteria HT115 (DE3) (Fire A, Carnegie Institution, Baltimore, MD) transformed with the plasmids. Individual nematodes at the L4  
20 growth stage were then placed in single wells at day 1. In each well 1 nematode (P1). At day two, the P1 nematodes were placed to a new well and left to incubate for a day. The same procedure was repeated at day 3. All plates were further incubated at 25°C to  
25 allow offspring to be formed. Sup35 RNAi induced survival (rescue) was measured by counting the offspring.

#### Results

30 RNAi experiment in *C. elegans* nuc-1/pha-1(e2123)ts mutants by feeding with *E. coli* expressing sup-35 dsRNA.

- 30 -

Set up:

pGN1 as control

pGN2 (sup 35 - Term.)

pGN12 (sup 35 + Term.)

5

pGN2 + pGN1 dilutions 1/2, 1/4, 1/8, 1/16, 1/32

pGN12 + pGN1 dilutions 1/2, 1/4, 1/8, 1/16, 1/32

10 Conditions:

Incubation temperature 25°C

Readout:

Count offspring (adult hermaphrodites)

pGN1 (control)

Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	0	0	0

pGN2 (undiluted)

Day 1	12	4	48	32
Day 2	24	23	80	85
Day 3	5	0	9	16

pGN12 (undiluted)

Day 1	16	29	37	14
Day 2	27	22	57	2
Day 3	1	2	4	1

pGN 2+1, 1/2 dilution

Day 1	0	7	0	2
Day 2	9	10	0	3
Day 3	0	2	0	0

pGN 12+1, 1/2 dilution

Day 1	22	28	103	61
Day 2	36	45	53	40
Day 3	3	3	25	1



- 31 -

pGN 2+1, 1/4 dilution

Day 1	28	23	0	0
Day 2	6	3	0	0
Day 3	0	0	0	0

pGN 12+1, 1/4 dilution

Day 1	*	6	36	5
Day 2		24	55	3
Day 3				

pGN 2+1, 1/8 dilution

Day 1	0	0	4	0
Day 2	0	0	11	0
Day 3	0	0	0	0

pGN 12+1, 1/8 dilution

Day 1	31	12	16	38
Day 2	4	5	37	4
Day 3	0	0	2	1

pGN 2+1, 1/16 dilution

Day 1	0	0	0	0
Day 2	0	0	0	1 little
Day 3	0	0	0	0

pGN 12+1, 1/16 dilution

Day 1	1	0	0	0
Day 2	2	0	0	1
Day 3	0	1	1	1

pGN 2+1, 1/32 dilution

Day 1	0	0	0	0
Day 2	0	0	0	0
Day 3	0	0	0	0

pGN 12+1, 1/32 dilution

Day 1	0	0	1	0
Day 2	0	L2	3	0
Day 3	2	0	L3- L4	0

5

\* mother died

### Conclusions

As expected, worms fed by bacteria harboring pGN1, did not result in the viable offspring, due to the lethal effect of the pha-1 mutation at this temperature.

5 Feeding the nematodes with *E. coli* harboring pGN2 or pGN12 both result in viable offspring. This is due to the feeding of the worm with dsRNA from sup-35. The remarkable difference between the two feeding experiments can be seen in the dilution series. When  
10 diluting the bacteria harboring pGN2 with bacteria harboring pGN1, the number of offspring diminishes drastically, even at a low dilution of one to two. This dilution series indicates that high levels of dsRNA are needed to have a proper RNAi induction. In  
15 the feeding experiment with bacteria harboring pGN12, significant offspring is still observed at a dilution of one to eight. This indicates that in the bacteria harboring pGN12, much more effective dsRNA is formed. This experiment clearly shows that the addition of  
20 terminator sequences in vectors to express dsRNA as described above provide a significant advantage in the generation of RNAi.

### Example 3: Comparison of RNAi efficiency of vectors 25 with and without T7 terminators(pGN49 vs pGN59)

Three different genes have been cloned in the vectors pGN49A and pGN59A. The cloning was performed by amplifying the gene fragments with PfuI DNA polymerase  
30 producing blunt ends, facilitating cloning in these vectors. These PCR fragments were cloned into the vectors digested with SrfI. Correct fragment insertion of the clones was checked by PCR. The fragments are chosen such that ds expression and RNAi results in a

lethal phenotype of the offspring. This procedure allows to compare fast and easy the efficiency of the two vectors pGN49 and pGN59 in RNAi.

plasmid	Gene (acedb)	Vector backbone5
pGW5	B0511.8	pGN49A
pGW9	C01G8.7	pGN49A
pGW11	C47B2.3	pGN49A
pGW17	B0511.8	pGN59A
pGW21	C01G8.7	pGN59A
pGW23	C47B2.3	pGN59A

All the plasmids (pGW-series) are transformed in *E. coli* AB301-105 (DE3) bacteria by standard methodology. The bacteria are then grown in LB/amp at 37°C for 14-18h.

25 These cultures were centrifuged and the bacterial pellet dissolved in S-complete buffer containing 1mM IPTG and 100 µg/µl ampiciline.

In 96 well plates containing 100 µl S-complete (containing 1 mM IPTG and 100 µg/µl ampiciline final concentration) and 10 µl of bacteria solution, 3

30 nematodes were added at each well, the nematodes were at the L1 growth stage.

The plates were incubated at 25°C for 5-6 days. Each

- 34 -

day the plates are inspected for development of the larvae and the production of F1 offspring.

## 5 Results

The RNAi was performed in eight-fold for each constructed plasmid. The results show that when T7 terminators are inserted into the vector backbone, the expected phenotype gives a 100% occurrence. When T7 terminators are not used the reproducibility can decrease up to 50%. As in the previous experiment, the results show that the addition of terminators significantly enhances RNAi performance.

### DNA

fragment	B0511.8	B0511.8	C01G8.7	C01G8.7	C47B2.3	C47B2.3
Vector	pGN49A	pGN59A	PGN49A	pGN59A	pGN49A	pGN59A
Resulting						
plasmid	PGW5	PGW17	PGW9	PGW21	PGW11	PGW23
Percentage						
lethal	100	75	100	87.5	100	50
Percentage						
offspring	0	25	0	12.5	0	50

15

PCR fragment generated by the primers oGN103 and  
oGN104 on template pCDM8

TACCAAGGCT AGCATGGTTT ATCACTGATA AGTTGG  
5 ATAAGTTGGT GGACATATTA TGTTTATCAG TGATAAAGTG TCAAGCATGA  
CAAAGTTGCA GCCGAATACA GTGATCCGTG CCGGCCCTGG ACTGTTGAAC  
GAGGTCGGCG TAGACGGTCT GACGACACGC AAAGTGGCGG AACGGTTGGG  
GGTGCAGCAG CCGGCGCTTT ACTGGCACTT CAGGAACAAG CGGGCGCTGC  
TCGACGCACT GGCCGAAGCC ATGCTGGCGG AGAATCATAC GCTTCGGTGC  
10 CGAGAGCCGA CGACGACTGG CGCTCATTTC TGATCGGGAA TCCCGCAGCT  
TCAGGCAGGC CCATGCTAGC CTTGGTACCA GCACAATGG

Overlap PCR Fragment, which was used to generate  
15 pGN49A

gatcttgatccggcttactaaaagccagataaacagtatgcgtatattgcgcgctg  
atttttgcggtataagaatatatactgatatgtatacccggaagtatgtcaaaa  
gagggtgtgctatgaagcagcgtattacagtgcaggttgacagcgacagctatca  
20 gttgctcaaggcatatatgatgtcaatatctcgggtctggttaagcacaacatg  
cagaatgaagcccgtcgtctgcgtgccgaacgctggaaagcggaaaatcaggaa  
gggatggctgaggtcgcccgggtttattgaaatgaacggctcttttgctgacgag  
aacagggactggtgaaatgcagtttaagggtttacacctataaaagagagagccg  
ttatcgtctgtttgtggatgtacagagtgatattattgacacgcccgggcga  
25 cggatggtgatccccctggccagtgcacgtctcttaagcgataaagtctccc  
gtgaactttaccgggtggtgcatatcggggatgaaagctggcgcatgatgac  
caccgatatggccagtgtgccggtctccgttatcggggaagaagtggctgat  
ctcagccaccgcgaaaatgacatcaaaaacgccattaacctgatgttctggg  
gaatataaatgtcaggctcccttatacacagc

30

**Claims:**

1. A DNA construct comprising:
  - a) a first promoter and
  - 5 b) a second promoter,  
in which the first and second promoter are in  
opposite orientation to each other and define:
    - c) an inter-promoter region positioned downstream of  
the 3' end of the first promoter and downstream of  
10 the 3' end of the second promoter;  
and which DNA construct further comprises:
      - d) at least one cloning site positioned in the inter-  
promoter region; and
      - e) a first transcription terminator, positioned (as  
15 seen from the 3' end of the first promoter)  
downstream of the first promoter and downstream of  
the at least one cloning site, wherein the first  
transcription terminator is operably linked to the  
first promoter.
- 20 2. A DNA construct according to claim 1, further  
comprising:
  - f) a second transcription terminator positioned (as  
seen from the 3' end of the second promoter)  
25 downstream of the second promoter and downstream of  
the at least one cloning site.  
wherein the second transcription terminator is  
operably linked to the second promoter.
- 30 3. A DNA construct according to claim 1 or 2, in  
which the first transcription terminator is  
positioned in the inter-promoter region.



4. A DNA construct according to claim 1 or 2, in which the first transcription terminator is positioned (as seen from the 3' end of the first promoter) downstream of the first promoter, downstream of the at least one cloning site, and downstream of the 5' end of the second promoter.
5. A DNA construct according to any one of claims 2, 3 or 4, in which the second transcription terminator is positioned in the inter-promoter region.
6. A DNA construct according to any of claims 2, 3 or 4 in which the second transcription terminator is positioned (as seen from the 3' end of the second promoter) downstream of the second promoter, downstream of the at least one cloning site, and downstream of the 5' end of the first promoter.
7. A DNA construct according to any one of claims 4, 5 or 6, in which the 3' end of the first transcription terminator is separated from the 5' end of the second promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides, particularly preferably no more than 10

nucleotides, more particularly preferably no more than 6 nucleotides.

- 5 8. A DNA construct according to any one of claims 6 or 7, in which the 3' end of the second transcription terminator is separated from the 5' end of the first promoter by no more than 2000 nucleotides, preferably no more than 1000 nucleotides, more preferably no more than 500  
10 nucleotides, even more preferably no more than 200 nucleotides, especially preferably no more than 100 nucleotides, more especially preferably no more than 50 nucleotides, even more especially preferably no more than 20 nucleotides,  
15 particularly preferably no more than 10 nucleotides, more particularly preferably no more than 6 nucleotides.
- 20 9. A construct according to any one of the preceding claims wherein the first and the second promoter are identical.
- 25 10. A DNA construct according to any one of the claims 1 to 7 wherein the first and the second promoter are non-identical.
- 30 11. A DNA construct according to claims 8 or 9 wherein the first promoter and the second promoter are independently chosen from T7, T3 or SP6 promoters.
12. A construct according to any one of the preceding claims wherein the cloning site comprises at

least one restriction site.

13. A DNA according to claim 11 wherein the cloning  
site comprises at least two restriction sites  
5 flanking a sequence of stuffer DNA.
14. A DNA construct according to claim 12 wherein the  
at least two restriction sites are identical.
- 10 15. A DNA construct according to any one of the claim  
12 to 13 wherein the at least one restriction  
site or the at least two restriction sites  
restriction sites are *BstXI* sites.
- 15 16. A DNA construct according to any one of the  
claims 12 to 13 wherein the restriction sites are  
*XcmI* sites.
- 20 17. A DNA construct according to any one of the  
preceding claims wherein the cloning site further  
comprises *attR1* and *attR2* recombination  
sequences.
- 25 18. A DNA construct according to any one of the  
preceding claims wherein the cloning site further  
comprises a *ccdB* nucleotide sequence.
- 30 19. A DNA construct according to claim 17 wherein the  
*ccdB* nucleotide sequence further comprises at  
least one unique restriction site.
20. A DNA construct according to claim 18 wherein the  
at least one unique restriction site are blunt-  
end restriction sites.

21. A DNA construct according to claim 19 wherein the blunt-end restriction sites are *SrfI* sites.
- 5 22. A DNA according to any one of the preceding claims which further comprises:  
g) a DNA fragment inserted in the at least one cloning site.
- 10 23. A DNA construct according to any one of the preceding claims which is a plasmid or vector.
24. A plasmid or vector as claimed in claim 23 having the nucleotide sequence illustrated in Figure 10,  
15 Figure 11, Figure 12, Figure 13, Figure 14, or Figure 15.
25. Use of the DNA construct according to any one of the preceding claims for the production of  
20 double-stranded RNA for RNA inhibition.
26. A bacterial strain harbouring the DNA construct according to any one of the preceding claims.
- 25 27. A bacterial strain according to claim 26, wherein said bacteria strain is an *E. coli* strain.
28. Use of the bacterial strain according to claims 26 or 27 for the production of double-stranded  
30 RNA for RNA inhibition.

FIG. 1(a)

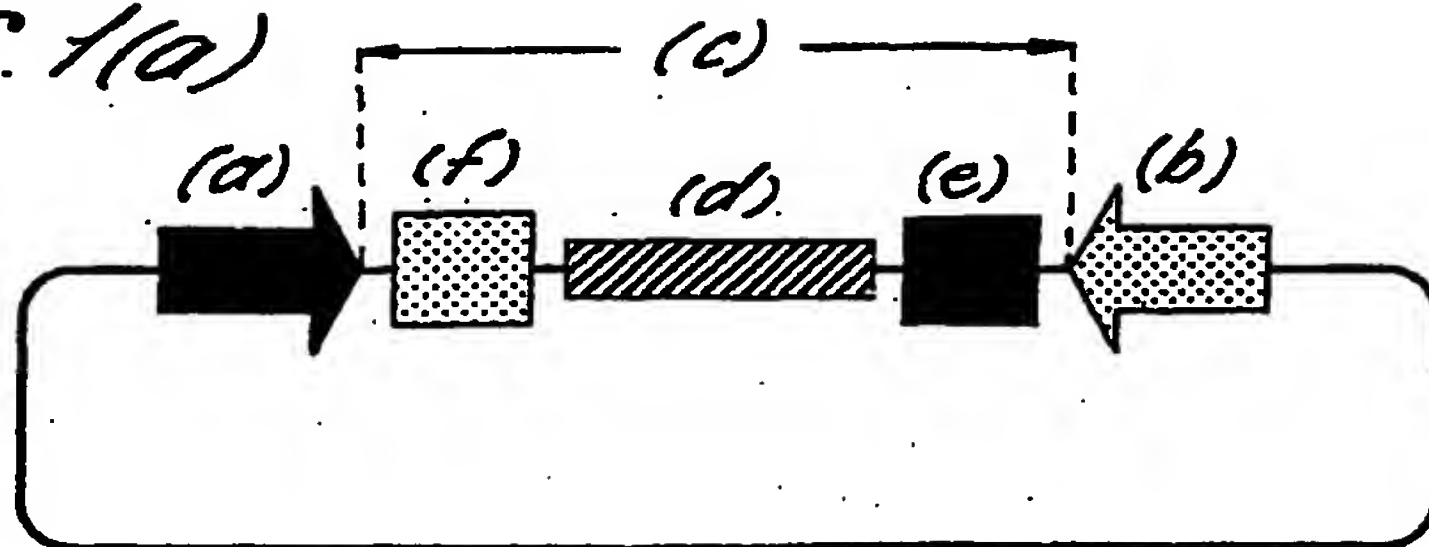


FIG. 1(b)

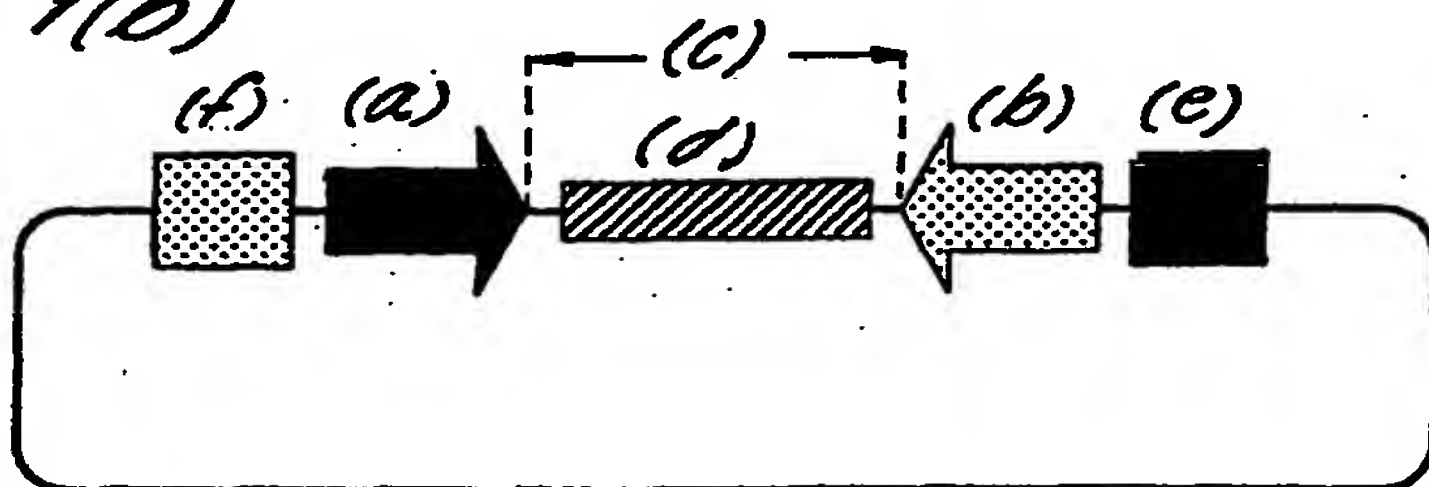
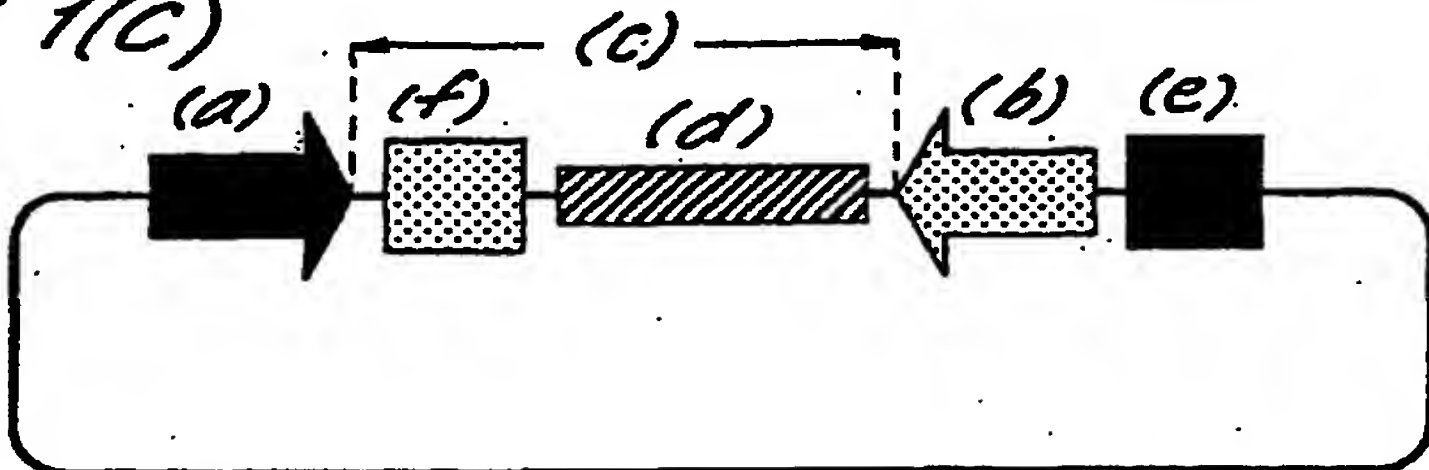


FIG. 1(c)



(a): promoter 1  
(b): promoter 2

(e): Terminator 1  
(f): Terminator 2

FIG. 1(d)

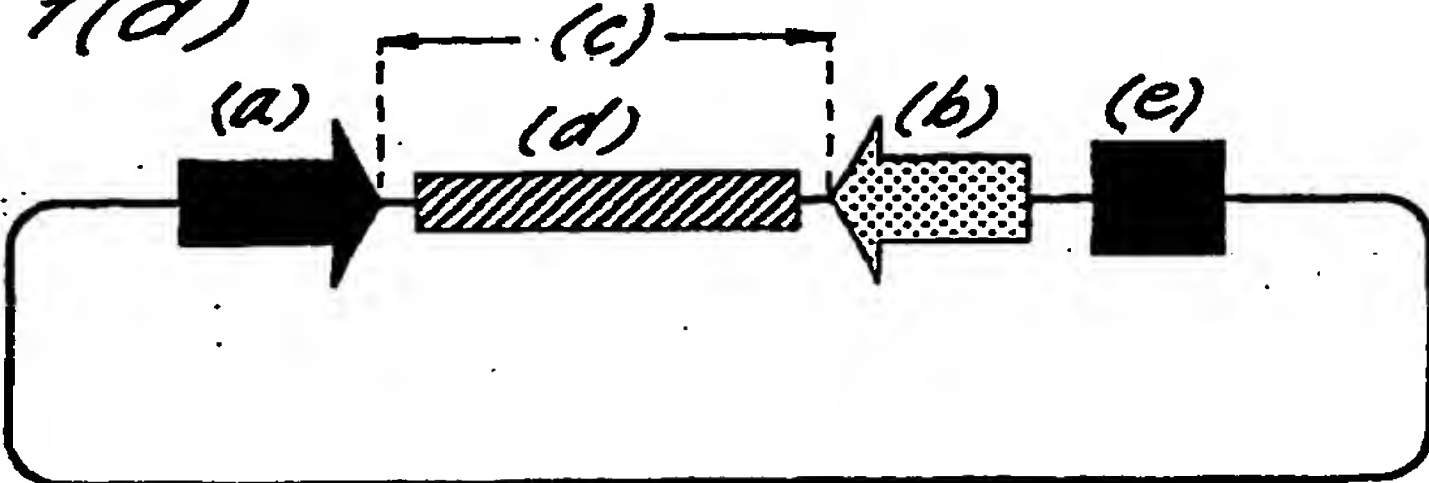


FIG. 1(e)

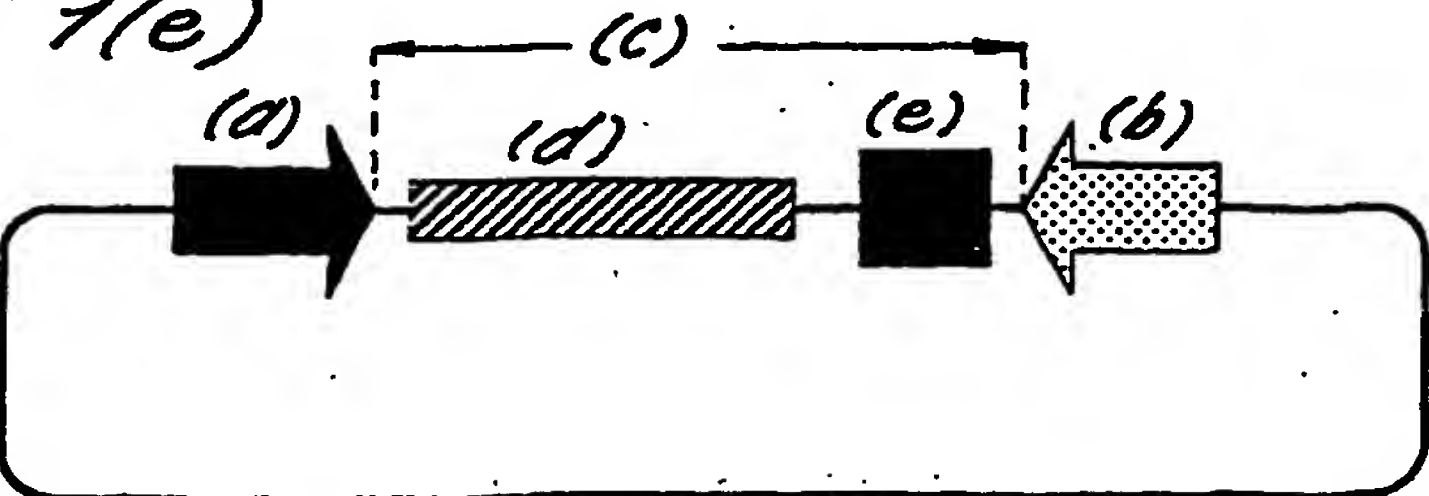


FIG. 2(a)

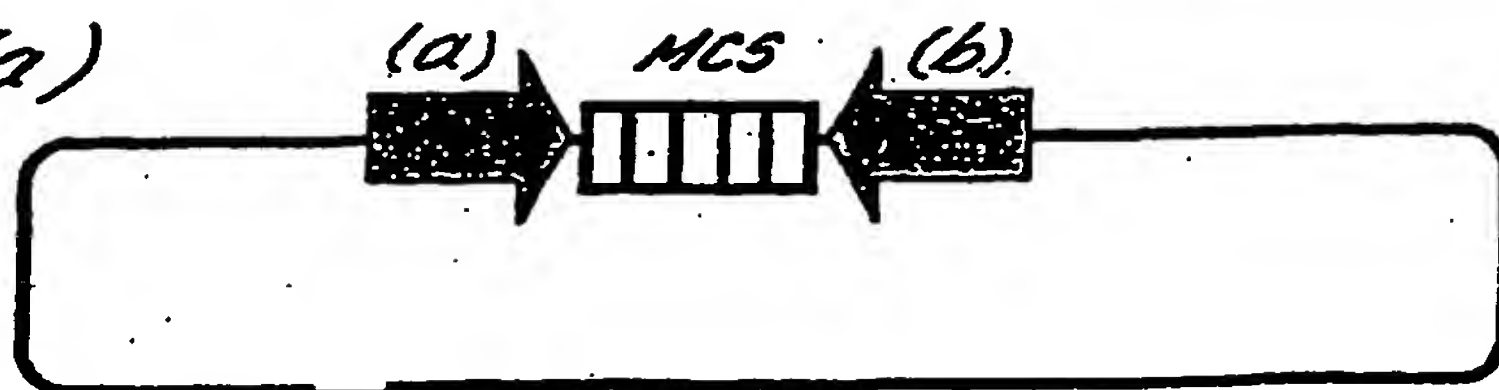


FIG. 2(b)

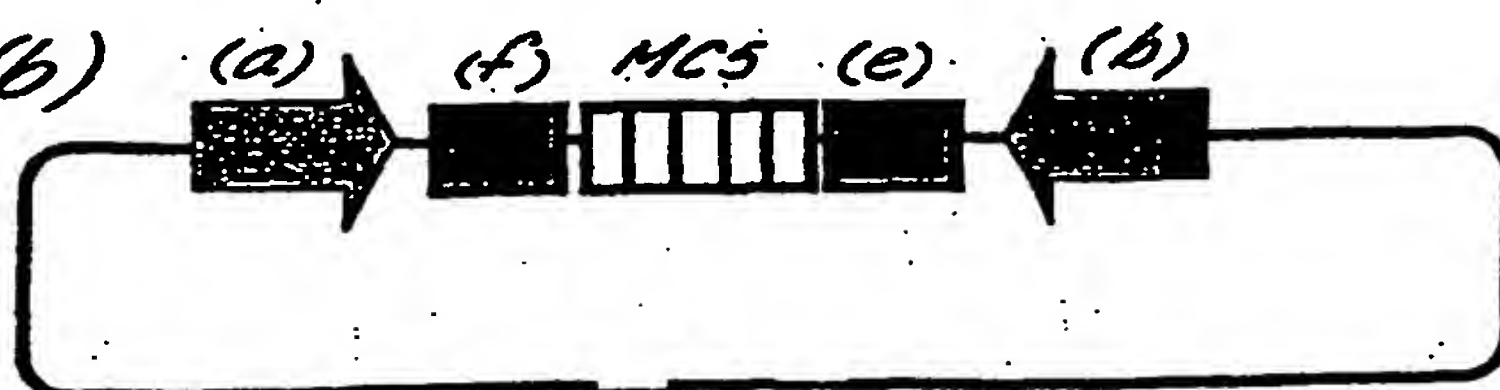


FIG. 2(c)

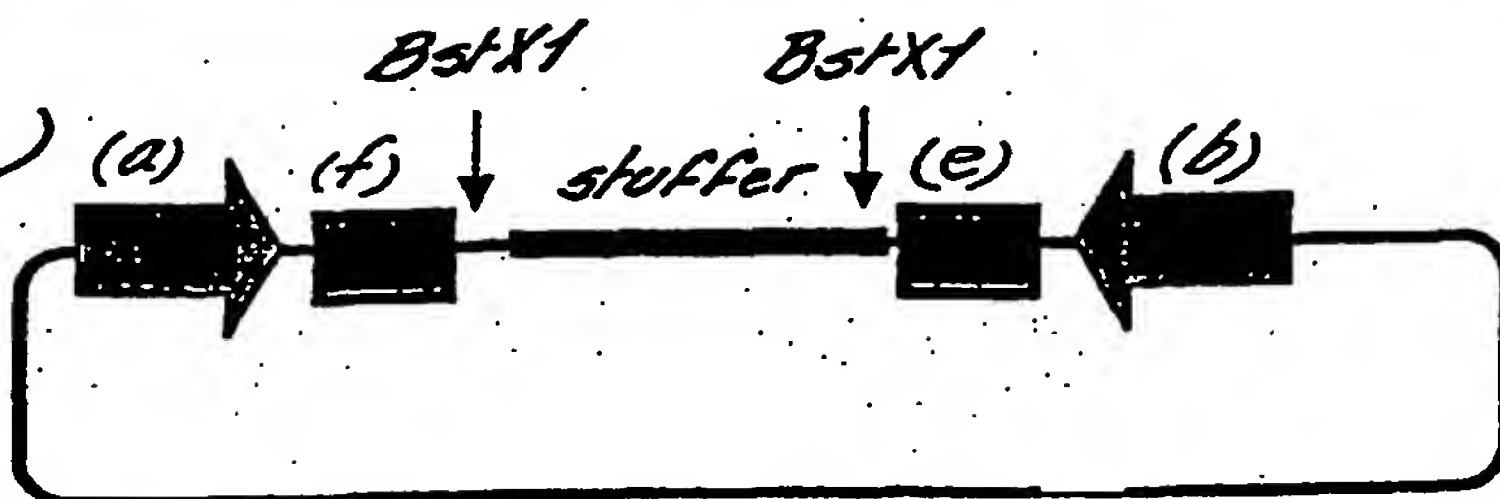


FIG. 2(d)

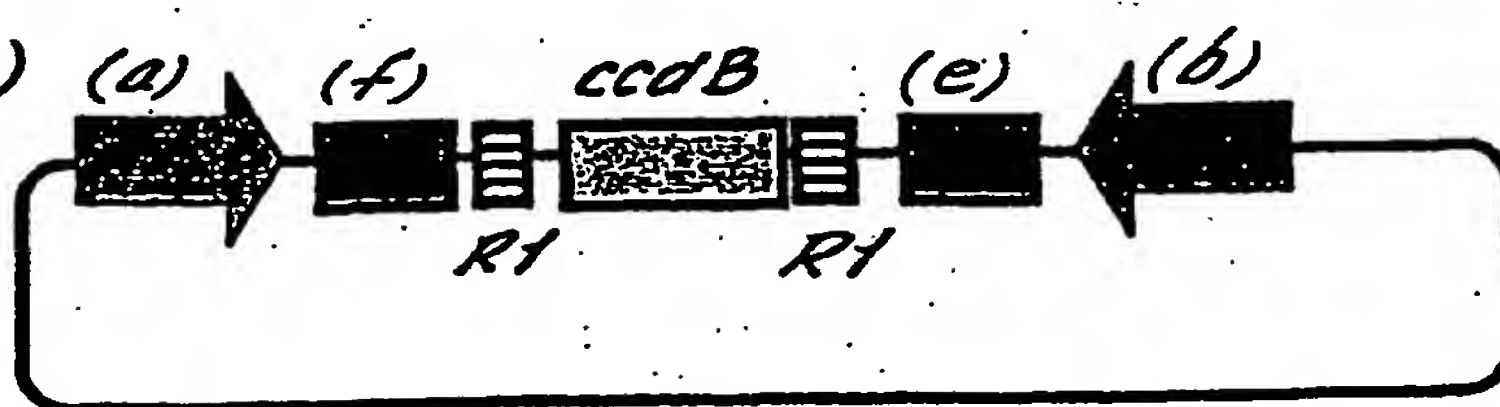


FIG. 2(e)

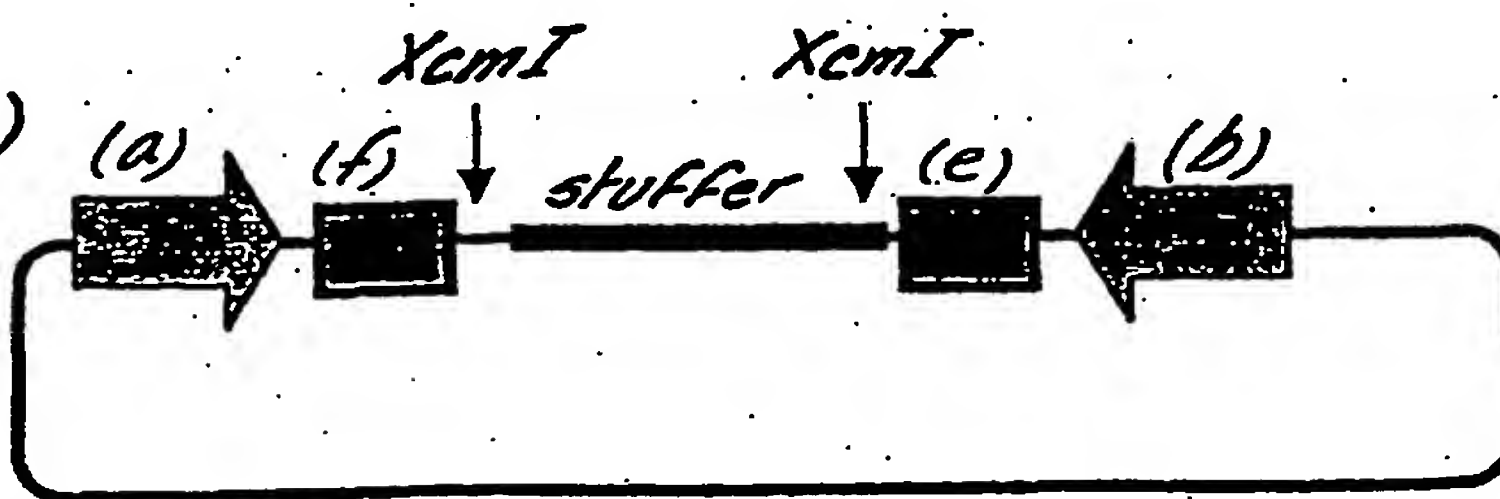
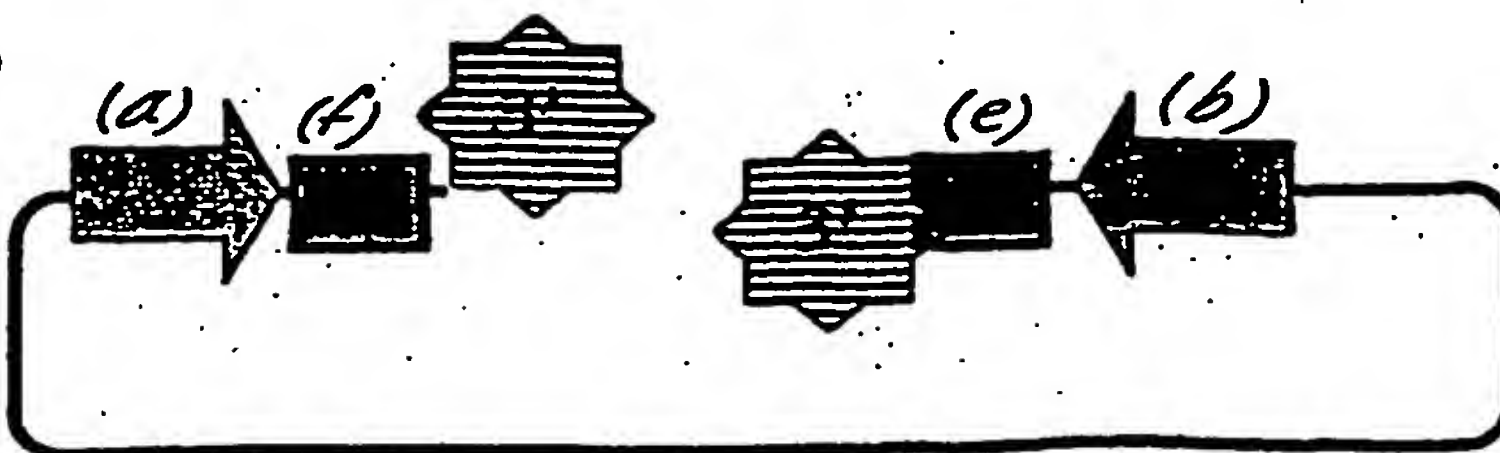


FIG. 2(f)





## Construction RNAi vector with T7 terminators

FIG. 3.

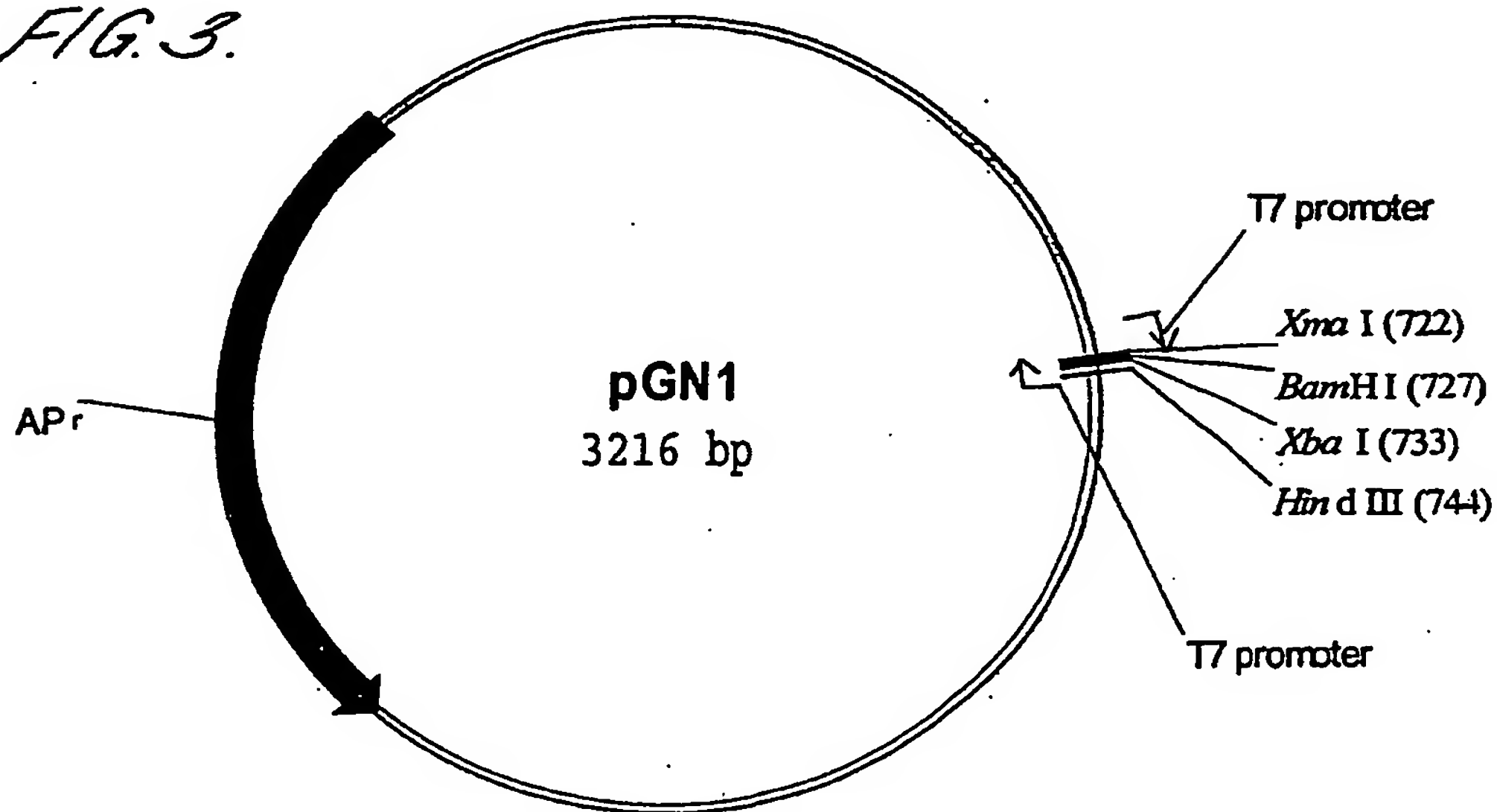


FIG. 4.

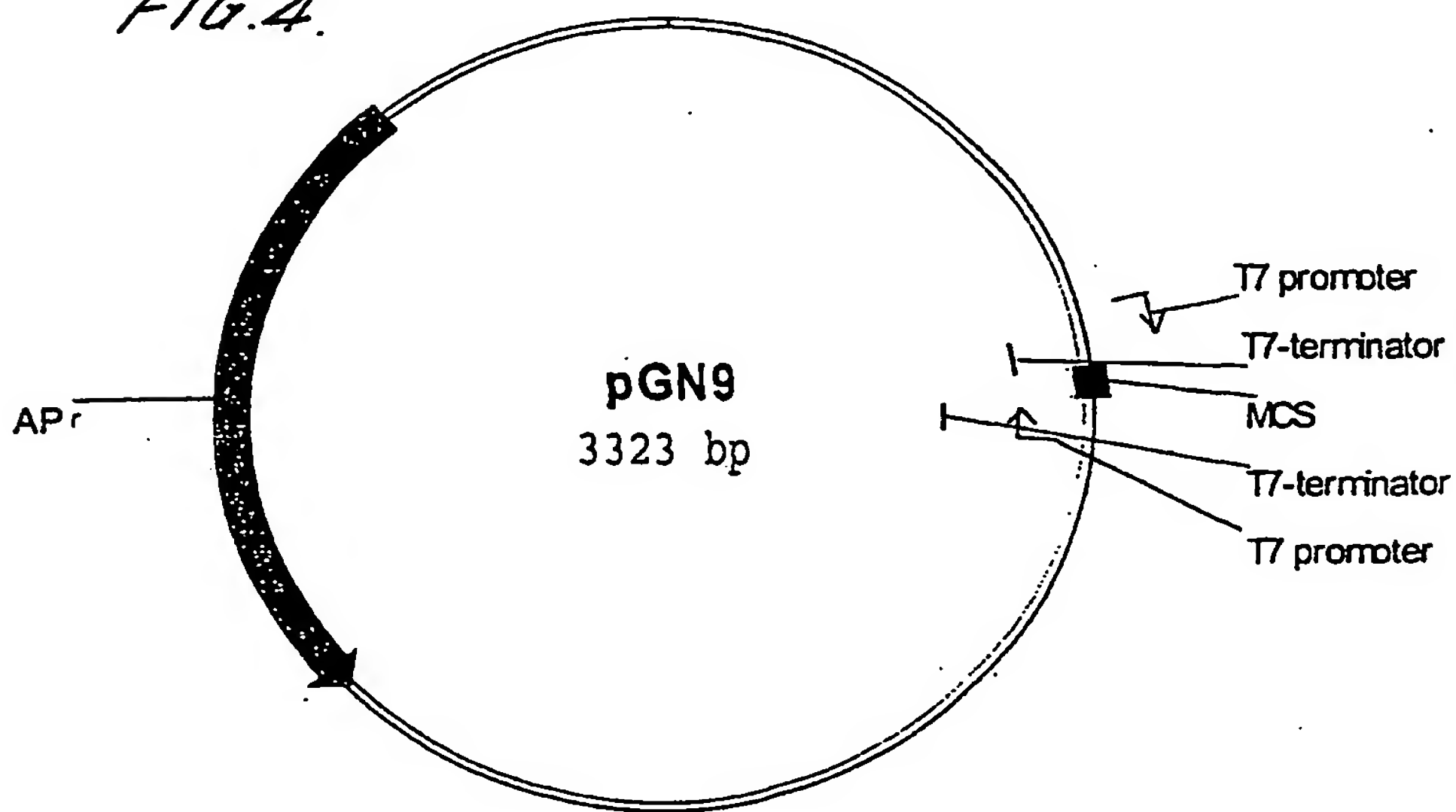


FIG. 5.

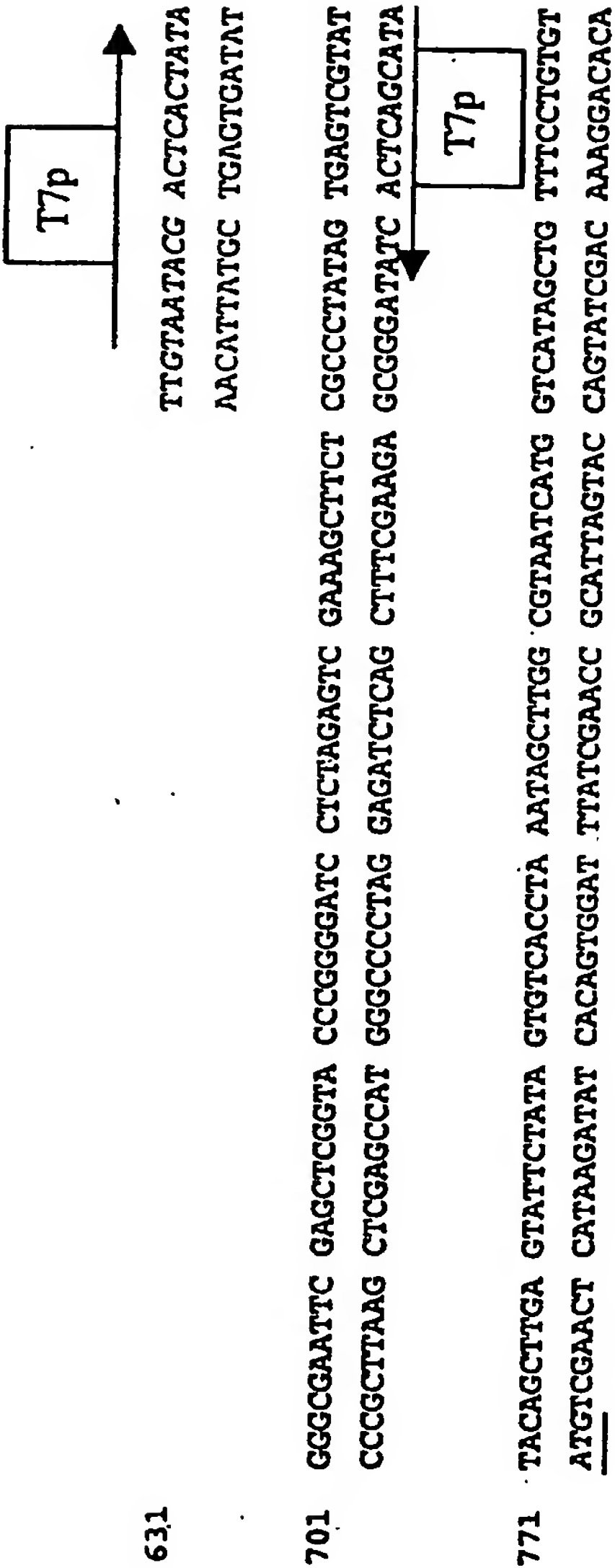


FIG. 6.

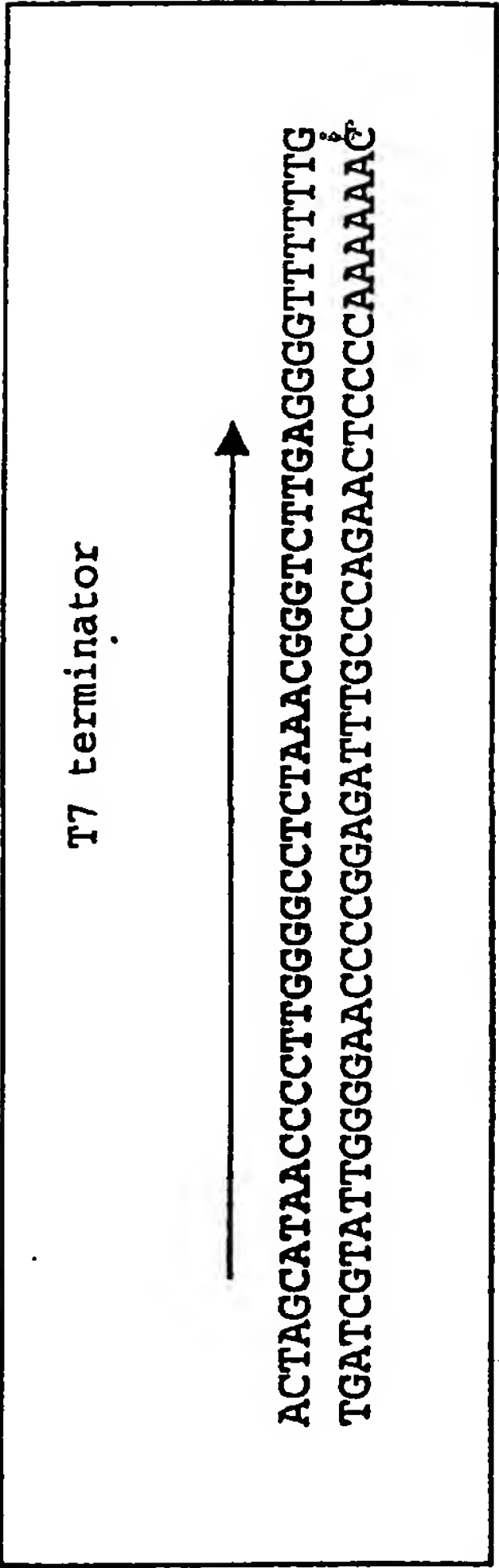


FIG. 7.

	T7 terminator	EcoRI PstI KpnI	
OGN27	5' AATTCAAAAACCCCTCAAGACCCGTTTAGAGGCCCCCAAGGGTTATGCTAGTGAATTCTGCAGCGGTAC		3'
OGN28	3' GTTTTTTGGGGAGTTCTGGGCAAATCTCCGGGGTTCCTCCCAATACGATCACTTAAGACGTCGC		5'
	↓		
	T7 terminator		
	XbaI MluI HindIII	HindIII com.	
OGN 29	5' CTAGACGCGTAAGCTTACTAGCATAAACCCTTGGGCCCTCTAAACGGGTCTTGAGGGGTTTTTG		3'
OGN 30	3' TGGCGCATTCGAAATGATCGTATTGGGGAAACCCCGGAGATTGCCCCAGAACTCCCCCAAAAACTCGA		5'

FIG. 8.

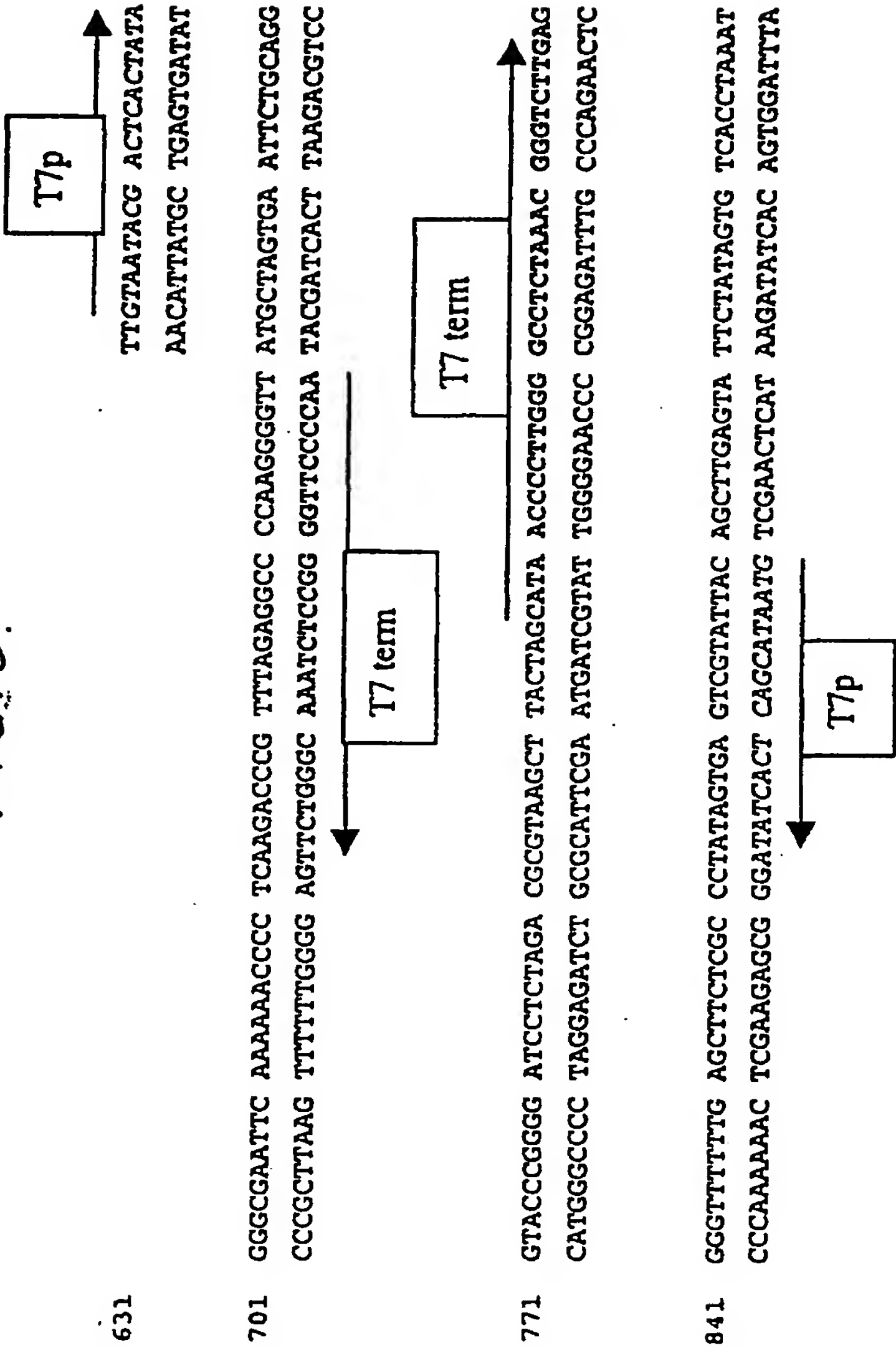
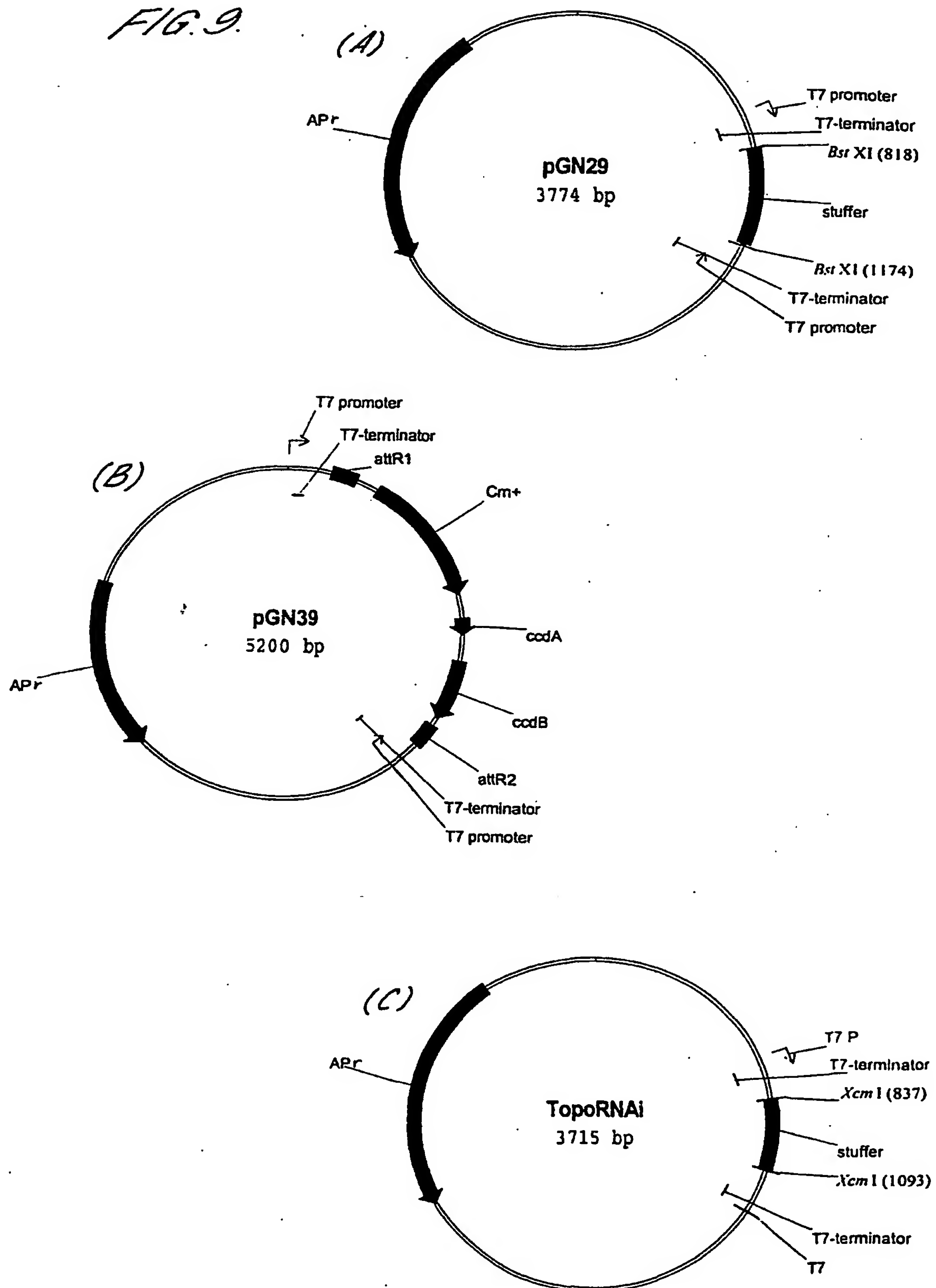


FIG. 9.



*FIG. 10.*

pGN9

```
1  gagtgcacca tatgcggtgt gaaataccgc acagatgcgt aaggagaaaa taccgcatca
61  ggcgaaattg taaacgttaa tattttgtta aaattcgcgt taaatatttg ttaaatacagc
121 tcatttttta accaataggg cgaaatcggc aaaatccctt ataaatcaaa agaatagacc
181 gagatagggg tgagtgttgt tccagtttgg aacaagagtc cactattaaa gaacgtggac
241 tccaacgtca aagggcgaaa aaccgtctat cagggcgatg gccactacg tgaaccatca
301 cccaaatcaa gttttttgcg gtcgaggtgc cgtaaagctc taaatcggaa ccctaaaggg
361 agcccccgat ttagagcttg acggggaaa ggcggcgaac tggcgagaaa ggaaggggag
421 aaagcgaaa gaggcggcgc tagggcgctg gcaagtgtag cggtcacgct gcgcgtaacc
481 accacacccg ccgcgcttaa tgcgcgcgta cagggcgctt ccattcgcca ttcaggctgc
541 gcaactgttg ggaagggcga tcggtgcggg cctcttcgct attacgccag ctggcgaaa g
601 ggggatgtgc tgcaaggcga ttaagttggg taacgccagg gttttccag tcacgacgtt
661 gtaaaacgac ggccagtga ttaataacg actcactata gggcgaattc aaaaaacccc
721 tcaagaccg tttagaggcc ccaaggggtt atgctagtga attctgcagg gtacccgggg
781 atcctctaga cgcgtaagct tactagcata accccttggg gcctctaaac gggctctgag
841 gggttttttg agcttctcgc cctatagtga gtcgtattac agcttgagta ttctatagtg
901 tcacctaaat agcttggcgt aatcatggtc atagctgttt cctgtgtgaa attgttatcc
961 gctcacaatt ccacacaaca tacgagccgg aagcataaag tgtaaagcct ggggtgccta
1021 atgagtgagc taactcacat taattgcgtt gcgctcactg cccgctttcc agtcgggaaa
1081 cctgtcgtgc cagctgcatt aatgaatcgg ccaacgcgcg gggagaggcg gtttgcgtat
1141 tgggcgctct tccgcttcc cgtcactga ctcgctgcgc tcggtcgttc ggctgcggcg
1201 agcggtatca gctcactcaa aggcggtaat acggttatcc acagaatcag gggataacgc
1261 aggaagaaac atgtgagcaa aaggccagca aaaggccagg aaccgtaaaa aggcgcgctt
1321 gctggcgttt ttcgataggg tccgcccccc tgacgagcat cacaaaaatc gacgctcaag
1381 tcagaggtgg cgaaaccgga caggactata aagataccag gcgtttcccc ctggaagctc
1441 cctcgtgcgc tctcctgttc cgaccctgcc gcttaccgga tacctgtccg cctttctccc
1501 ttcgggaagc gtggcgcttt ctcatagctc acgctgtagg tatctcagtt cgggtgtaggt
1561 cgttcgctcc aagctgggct gtgtgcacga acccccctt cagcccgacc gctgcgcctt
1621 atccggtaac tatcgtcttg agtccaaccc ggtaagacac gacttatcgc cactggcagc
1681 agccactggt aacaggatta gcagagcgag gtatgtaggc ggtgctacag agttcttgaa
1741 gtggtggcct aactacggct acactagaag gacagtattt ggtatctgcg ctctgctgaa
1801 gccagttacc ttcggaaaaa gagttggtag ctcttgatcc ggcaaaaaa ccaccgctgg
1861 tagcgggtgt ttttttgttt gcaagcagca gattacgcgc agaaaaaaag gatctcaaga
1921 agatcctttg atcttttcta cggggtctga cgtcagttgg aacgaaaact cacgttaagg
1981 gatttttggtc atgagattat caaaaaggat cttcacctag atccttttaa attaaaaatg
2041 aagtttttaa tcaatctaaa gtatatatga gtaaacttgg tctgacagtt accaatgctt
2101 aatcagtgag gcacctatct cagcgatctg tctatttcgt tcatccatag ttgcctgact
2161 ccccgctcgt tagataacta cgatacggga gggcttacca tctggcccca gtgctgcaat
2221 gataccgcga gacccacgct caccggctcc agatttatca gcaataaacc agccagccgg
2281 aagggccgag cgcagaagtg gtcttgcaac tttatccgcc tccatccagt ctattaattg
2341 ttgccgggaa gctagagtaa gtagttcgcc agttaatagt ttgcgcaacg ttgttggcat
2401 tgctacaggg atcgtggtgt cacgctcgtc gtttggtatg gcttcattca gctccggttc
2461 ccaacgatca aggcgagtta catgatcccc catgttgtgc aaaaaagcgg ttagctcctt
2521 cggtcctccg atcgttgtca gaagtaagtt ggccgcagtg ttatcactca tgggtatggc
2581 agcactgcat aattctctta ctgtcatgcc atccgtaaga tgcttttctg tgactggtga
2641 gtactcaacc aagtcattct gagaataacc cgcccgcgca ccgagttgct cttgcccggc
2701 gtcaatacgg gataatagtg tatgacatag cagaacttta aaagtgctca tcattggaaa
2761 acgttcttcg gggcgaaaac tctcaaggat cttaccgctg ttgagatcca gttcgatgta
2821 acccactcgt gcacccaact gatcttcagc atcttttact ttcaccagcg tttctgggtg
2881 agcaaaaaa ggaaggcaaa atgccgcaaa aaaggggaata agggcgacac ggaaatgttg
2941 aatactcata ctcttccttt ttcaatatta ttgaagcatt tatcagggtt attgtctcat
3001 gagcggatac atatttgaat gtatttagaa aaataaacia ataggggttc cgcgcacatt
3061 tccccgaaaa gtgccacctg acgtctaaga aaccattatt atcatgacat taacctataa
3121 aaataggcgt atcacgaggc cctttcgtct cgcgcgtttc ggtgatgacg gtgaaaacct
3181 ctgacacatg cagctcccgg agacgggtcac agcttgtctg taagcggatg ccgggagcag
3241 acaagcccgt cagggcgctt cagcgggtgt tggcgggtgt cggggctggc ttaactatgc
3301 ggcatacagag cagattgtac tga
```



## FIG. 11.

PGN29

```
1  gagtgcacca  tatgcggtgt  gaaataccgc  acagatgcgt  aaggagaaaa  taccgcatca
61  ggcgaaattg  taaacgttaa  tattttgtta  aaattcgcgt  taaatatttg  ttaaatcagc
121  tcatttttta  accaataggc  cgaaatcggc  aaaatccctt  ataaatcaaa  agaatagacc
181  gagatagggg  tgagtgttgt  tccagtttgg  aacaagagtc  cactattaaa  gaacgtggac
241  tccaacgtca  aagggcgaaa  aaccgtctat  cagggcgatg  gccactacg  tgaaccatca
301  cccaaatcaa  gttttttgcg  gtcgaggtgc  cgtaaagctc  taaatcggaa  ccctaaaggg
361  agccccgat  ttagagcttg  acggggaaa  ccggcgaaac  tggcgagaaa  ggaaggggag
421  aaagcgaaag  gagcggggcg  tagggcgctg  gcaagtgtag  cggtcacgct  gcgcgtaacc
481  accacacccg  ccgcgcttaa  tgcgccgcta  cagggcgctg  ccattcgcca  ttcaggctgc
541  gcaactgttg  ggaagggcga  tcgggtgcgg  cctcttcgct  attacgccag  ctggcgaaag
601  ggggatgtgc  tgcaaggcga  ttaagttggg  taacgccagg  gttttccag  tcacgacgtt
661  gtaaaacgac  ggccagtga  ttgtaatacg  actcactata  gggcgaaatc  aaaaaacccc
721  tcaagacccg  ttagaggcc  ecaaggggtt  atgctagtga  attctgcagg  gtaccggggg
781  atcctctaga  gatccctcga  cctcgagatc  cattgtgctg  gcgcggattc  tttatcactg
841  ataagttgg  ggacatatta  tgtttatcag  tgataaagt  tcaagcatga  caaagttgca
901  gccgaataca  gtgatccgtg  ccggccctgg  actgttgaac  gaggtcggcg  tagacgggtc
961  gacgacacgc  aaactggcgg  aacggttggg  ggtgcagcag  ccggcgcttt  actggcactt
1021  caggaacaag  cgggcgctgc  tcgacgcact  ggccgaagcc  atgctggcgg  agaatcatac
1081  gcttcgggtg  cgagagccga  cgacgactgg  cgctcatttc  tgatcgggaa  tcccgcagct
1141  tcaggcaggc  gctgctcgcc  taccgccagc  acaatggatc  tcgagggatc  ttccatacct
1201  accagttctg  cgctgcagg  tcggcgccgc  gactctctag  acgcgtaagc  ttactagcat
1261  aacccttgg  ggctctaaa  cgggtcttga  ggggtttttt  gagcttctcg  ccctatagtg
1321  agtcgtatta  cagcttgagt  attctatagt  gtcacctaaa  tagcttggcg  taatcatggg
1381  catagctgtt  tcctgtgtga  aattgttatc  cgctcacaat  tccacacaac  atacgagccg
1441  gaagcataaa  gtgtaaagcc  tggggtgcct  aatgagtga  ctaactcaca  ttaattgcgt
1501  tgcgctcact  gcccgctttc  cagtcgggaa  acctgtcgtg  ccagctgcat  taatgaatcg
1561  gccaacgcgc  ggggagaggc  ggtttgcgta  ttgggcgctc  ttccgcttcc  tcgctcactg
1621  actcgctgcg  ctcggtcggt  cggtgcggc  gagcgggtat  agctcactca  aaggcggtaa
1681  tacggttatc  cacagaatca  ggggataacg  caggaaagaa  catgtgagca  aaaggccagc
1741  aaaaggccag  gaaccgtaaa  aaggccgcgt  tgctggcggt  tttcgatagg  ctccgcccc
1801  ctgacgagca  tcacaaaaat  cgacgctcaa  gtcagagggt  gcgaaacccg  acaggactat
1861  aaagatacca  ggcgtttccc  cctggaagct  cctcgtgcg  ctctcctgtt  ccgacctgc
1921  cgcttacccg  atacctgtcc  gcctttctcc  cttcggaag  cgtggcgctt  tctcatagct
1981  cacgctgtag  gtatctcagt  tcggtgtagg  tcgttcgctc  caagctgggc  tgtgtgcagc
2041  aacccccctg  tcagcccagc  cgctgcgcct  tatccggtaa  ctatcgctct  gagtccaacc
2101  cggtaagaca  cgacttatcg  ccactggcag  cagccactgg  taacaggatt  agcagagcga
2161  ggtatgtagg  cggtgctaca  gagttcttga  agtggtggcc  taactacggc  tacactagaa
2221  ggacagtatt  tggatatctg  gctctgctga  agccagttac  cttcgaaaa  agagttggta
2281  gctcttgatc  cggcaaaaca  accaccgtg  gtacgggtgg  tttttttgtt  tgcaagcagc
2341  agattacgcg  cagaaaaaaa  ggatctcaag  aagatccctt  gatcttttct  acggggtctg
2401  acgctcagtg  gaacgaaaac  tcacgttaag  ggatttttgt  catgagatta  tcaaaaagga
2461  tcttcaccta  gatcctttta  aattaaaaat  gaagttttaa  atcaatctaa  agtatatatg
2521  agtaaaactt  gtctgacagt  taccaatgct  taatcagtga  ggcacctatc  tcagcgatct
2581  gtctatttct  ttcattccata  gttgcctgac  tcccgcgtcg  gtagataact  acgatacggg
2641  agggcttacc  atctggcccc  agtgctgcaa  tgataccgcg  agaccacgc  tcaccggctc
2701  cagatttatc  agcaataaac  cagccagccg  gaagggccga  gcgcagaagt  ggtcctgcaa
2761  ctttatccgc  ctccatccag  tctattaatt  gttgccggga  agctagagta  agtagttcgc
2821  cagttaatag  tttgcgcaac  gttgttggca  ttgctacagg  catcgtggtg  tcacgctcgt
2881  cgtttgggat  ggcttcattc  agctccggtt  cccaacgata  aaggcgagtt  acatgatccc
2941  ccatgttgtg  caaaaaagcg  gttagctcct  tcggtcctcc  gatcgttgtc  agaagtaagt
3001  tggccgcagt  gttatcactc  atggttatgg  cagcactgca  taattctctt  actgtcatgc
3061  catccgtaag  atgcttttct  gtgactgggt  agtactcaac  caagtcattc  tgagaatacc
3121  gcgcccggcg  accgagttgc  tcttgcccgg  cgtcaatacg  ggataatagt  gtatgacata
3181  gcagaacttt  aaaagtgtc  atcattggaa  aacgttcttc  ggggcgaaaa  ctctcaagga
3241  tcttacccgt  gttgagatcc  agttcgatgt  aacccactcg  tgcacccaac  tgatcttcag
3301  catcttttac  tttcaccagc  gtttctgggt  gagcaaaaac  aggaaggcaa  aatgccgcaa
3361  aaaagggaat  aagggcgaca  cggaaatgtt  gaatactcat  actcttctct  tttcaatatt
3421  attgaagcat  ttatcagggt  tattgtctca  tgagcggata  catatttgaa  tgtatttaga
3481  aaaataaaca  aataggggtt  ccgcgcacat  ttccccgaaa  agtgccacct  gacgtctaag
3541  aaaccattat  tatcatgaca  ttaacctata  aaaataggcg  tatcacgagg  ccctttcgtc
3601  tcgcgcgttt  cggtgatgac  ggtgaaaacc  tctgacacat  gcagctcccg  gagacggtca
3661  cagcttgtct  gtaagcggat  gccgggagca  gacaagcccg  tcagggcgcg  tcagcgggtg
3721  ttggcgggtg  tcggggctgg  cttactatg  cggcatcaga  gcagattgta  ctga
```

*FIG. 12.**PGN39*

TAATACGACT CACTATAGGG CGAATTCAAA AAACCCCTCA AGACCCGTTT  
AGAGGCCCCA AGGGGTATG CTAGTGAATT CTGCAGCGGT ACCCGGGGAT  
CCTCTAGAGA TCCCTCGACC TCGAGATCCA TTGTGCTGGA AAGATCACAA  
GTTTGTACAA AAAAGCTGAA CGAGAAACGT AAAATGATAT AAATATCAAT  
ATATTAAATT AGATTTTGCA TAAAAACAG ACTACATAAT ACTGTAAAC  
ACAACATATC CAGTCACTAT GCGGCCGCA TTAGGCACCC CAGGCTTTAC  
ACTTTATGCT TCCGGCTCGT ATAATGTGTG GATTTTGAGT TAGGATCCGG  
CGAGATTTTC AGGAGCTAAG GAAGCTAAAA TGGAGAAAAA AATCACTGGA  
TATACCACCG TTGATATATC CCAATGGCAT CGTAAAGAAC ATTTTGAGGC  
ATTTCACTCA GTTGCTCAAT GTACCTATAA CCAGACCGTT CAGCTGGATA  
TTACGGCCTT TTTAAAGACC GTAAAGAAAA ATAAGCACAA GTTTTATCCG  
GCCTTTATTC ACATTCTTGC CCGCCTGATG AATGCTCATC CGGAATTCCG  
TATGGCAATG AAAGACGGTG AGCTGGTGAT ATGGGATAGT GTTCACCCTT  
GTTACACCGT TTTCCATGAG CAACTGAAA CGTTTTCATC GCTCTGGAGT  
GAATACCACG ACGATTTCCG GCAGTTTCTA CACATATATT CGCAAGATGT  
GGCGTGTTAC GGTGAAAACC TGGCCTATTT CCTAAAGGG TTTATTGAGA  
ATATGTTTTT CGTCTCAGCC AATCCCTGGG TGAGTTTCAC CAGTTTGTAT  
TTAAACGTGG CCAATATGGA CAACTTCTTC GCCCCGTTT TCACCATGGG  
CAAATATTAT ACGCAAGGCG ACAAGGTGCT GATGCCGCTG GCGATTCAGG  
TTCATCATGC CGTCTGTGAT GGCTTCCATG TCGGCAGAAT GCTTAATGAA  
TTACAACAGT ACTGCGATGA GTGGCAGGGC GGGGCGTAAA GATCTGGATC  
CGGCTTACTA AAAGCCAGAT AACAGTATGC GTATTTGCGC GCTGATTTTT  
GCGGTATAAG AATATATACT GATATGTATA CCCGAAGTAT GTCAAAAAGA  
GGTGTGCTAT GAAGCAGCGT ATTACAGTGA CAGTTGACAG CGACAGCTAT  
CAGTTGCTCA AGGCATATAT GATGTCAATA TCTCCGGTCT GGTAAGCACA  
ACCATGCAGA ATGAAGCCCG TCGTCTGCGT GCCGAACGCT GGAAAGCGGA  
AAATCAGGAA GGGATGGCTG AGGTGCCCCG GTTTATTGAA ATGAACGGCT  
CTTTTGCTGA CGAGAACAGG GACTGGTGAA ATGCAGTTTA AGGTTTACAC  
CTATAAAAGA GAGAGCCGTT ATCGTCTGTT TGTGGATGTA CAGAGTGATA  
TTATTGACAC GCCCGGGCGA CGGATGGTGA TCCCCCTGGC CAGTGCACGT  
CTGCTGTCAG ATAAAGTCTC CCGTGAACCT TACCCGGTGG TGCATATCGG  
GGATGAAAGC TGGCGCATGA TGACCACCGA TATGGCCAGT GTGCCGGTCT  
CCGTTATCGG GGAAGAAGTG GCTGATCTCA GCCACCGCGA AAATGACATC  
AAAAACGCCA TTAACCTGAT GTTCTGGGGA ATATAAATGT CAGGCTCCCT  
TATACACAGC CAGTCTGCAG GTCGACCATA GTGACTGGAT ATGTTGTGTT  
TTACAGTATT ATGTAGTCTG TTTTTTATGC AAAATCTAAT TTAATATATT  
GATATTTATA TCATTTTACG TTTCTCGTTC AGCTTTCTTG TACAAAGTGG  
TGATCTTTCC AGCACAATGG ATCTCGAGGG ATCTTCCATA CCTACCAGTT  
CTGCGCCTGC AGGTGCGGGC CGCGACTCTA GACGCGTAAG CTTACTAGCA  
TAACCCCTTG GGGCCTCTAA ACGGGTCTTG AGGGGTTTTT TGAGCTTCTC  
GCCCTATAGT GAGTCGTATT ACAGCTTGAG TATTCTATAG TGTCACCTAA  
ATAGCTTGGC GTAATCATGG TCATAGCTGT TTCCTGTGTG AAATTGTTAT  
CCGCTCACAA TTCCACACAA CATACGAGCC GGAAGCATAA AGTGTAAAGC

*FIG. 12 (CONTINUED 1)*

CTGGGGTGCC TAATGAGTGA GCTAACTCAC ATTAATTGCG TTGCGCTCAC  
TGCCCGCTTT CCAGTCGGGA AACCTGTCTG GCCAGCTGCA TTAATGAATC  
GGCCAACGCG CGGGGAGAGG CGGTTTGCGT ATTGGGCGCT CTTCCGCTTC  
CTCGCTCACT GACTCGCTGC GCTCGGTCTG TCGGCTGCGG CGAGCGGTAT  
CAGCTCACTC AAAGGCGGTA ATACGGTTAT CCACAGAATC AGGGGATAAC  
GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA GGAACCGTAA  
AAAGGCCGCG TTGCTGGCGT TTTTCGATAG GCTCCGCCCC CCTGACGAGC  
ATCACAAAAA TCGACGCTCA AGTCAGAGGT GGCGAAACCC GACAGGACTA  
TAAAGATACC AGGCGTTTCC CCCTGGAAGC TCCCTCGTGC GCTCTCCTGT  
TCCGACCCTG CCGCTTACCG GATACCTGTC CGCCTTTCTC CCTTCGGGAA  
GCGTGGCGCT TTCTCATAGC TCACGCTGTA GGTATCTCAG TTCGGTGTAG  
GTCGTTGCGT CCAAGCTGGG CTGTGTGCAC GAACCCCCCG TTCAGCCCGA  
CCGCTGCGCC TTATCCGGTA ACTATCGTCT TGAGTCCAAC CCGGTAAGAC  
ACGACTTATC GCCACTGGCA GCAGCCACTG GTAACAGGAT TAGCAGAGCG  
AGGTATGTAG GCGGTGCTAC AGAGTTCTTG AAGTGGTGGC CTAACACGG  
CTACACTAGA AGGACAGTAT TTGGTATCTG CGCTCTGCTG AAGCCAGTTA  
CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT CCGGCAAACA AACCACCGCT  
GGTAGCGGTG GTTTTTTTTGT TTGCAAGCAG CAGATTACGC GCAGAAAAAA  
AGGATCTCAA GAAGATCCTT TGATCTTTTC TACGGGGTCT GACGCTCAGT  
GGAACGAAAA CTCACGTAA GGGATTTTGG TCATGAGATT ATCAAAAAGG  
ATCTTCACCT AGATCCTTTT AAATTAATAA TGAAGTTTAA AATCAATCTA  
AAGTATATAT GAGTAACTT GGTCTGACAG TTACCAATGC TTAATCAGTG  
AGGCACCTAT CTCAGCGATC TGTCTATTTT GTTCATCCAT AGTTGCCTGA  
CTCCCCGTCG TGTAGATAAC TACGATACGG GAGGGCTTAC CATCTGGCCC  
CAGTGCTGCA ATGATACCGC GAGACCCACG CTCACCGGCT CCAGATTTAT  
CAGCAATAAA CCAGCCAGCC GGAAGGGCCG AGCGCAGAAG TGGTCCTGCA  
ACTTTATCCG CCTCCATCCA GTCTATTAAT TGTGCGCGG AAGCTAGAGT  
AAGTAGTTCG CCAGTTAATA GTTTGCGCAA CGTTGTTGGC ATTGCTACAG  
GCATCGTGGT GTCACGCTCG TCGTTTGGTA TGGCTTCATT CAGCTCCGGT  
TCCCAACGAT CAAGGCGAGT TACATGATCC CCCATGTTGT GCAAAAAGC  
GGTTAGCTCC TTCGGTCCTC CGATCGTTGT CAGAAGTAAG TTGGCCGCAG  
TGTTATCACT CATGGTTATG GCAGCACTGC ATAATTCTCT TACTGTCATG  
CCATCCGTAA GATGCTTTTC TGTGACTGGT GAGTACTCAA CCAAGTCATT  
CTGAGAATAC CGCGCCCGGC GACCGAGTTG CTCTTGCCCG GCGTCAATAC  
GGGATAATAG TGTATGACAT AGCAGAACTT TAAAAGTGCT CATCATTGGA  
AAACGTTCTT CGGGGCGAAA ACTCTCAAGG ATCTTACCGC TGTGAGATC  
CAGTTCGATG TAACCCACTC GTGCACCCAA CTGATCTTCA GCATCTTTTA  
CTTTCACCAG CGTTTCTGGG TGAGCAAAAA CAGGAAGGCA AAATGCCGCA  
AAAAAGGGAA TAAGGGCGAC ACGGAAATGT TGAATACTCA TACTCTTCCT  
TTTTCAATAT TATTGAAGCA TTTATCAGGG TTATTGTCTC ATGAGCGGAT  
ACATATTTGA ATGTATTTAG AAAAATAAAC AAATAGGGGT TCCGCGCACA  
TTTCCCCGAA AAGTGCCACC TGACGTCTAA GAAACCATTA TTATCATGAC  
ATTAACCTAT AAAAATAGGC GTATCACGAG GCCCTTTCGT CTCGCGCGTT  
TCGGTGATGA CGGTGAAAAC CTCTGACACA TGCAGCTCCC GGAGACGGTC  
ACAGCTTGTC TGTAAGCGGA TGCCGGGAGC AGACAAGCCC GTCAGGGCGC  
GTCAGCGGGT GTTGGCGGGT GTCGGGGCTG GCTTAACTAT GCGGCATCAG

*FIG. 12 (CONTINUED 2)*

AGCAGATTGT ACTGAGAGTG CACCATATGC GGTGTGAAAT ACCGCACAGA  
TGC GTAAGGA GAAAATACCG CATCAGGCGA AATTGTAAAC GTTAATATTT  
TGTTAAAATT CGCGTTAAAT ATTTGT TAAA TCAGCTCATT TTTTAACCAA  
TAGGCCGAAA TCGGCAAAAT CCCTTATAAA TCAAAAGAAT AGACCGAGAT  
AGGGTTGAGT GTTGTTCCAG TTTGGAACAA GAGTCCACTA TTAAAGAACG  
TGGACTCCAA CGTCAAAGGG CGAAAAACCG TCTATCAGGG CGATGGCCCA  
CTACGTGAAC CATCACCCAA ATCAAGTTTT TTGCGGTCGA GGTGCCGTAA  
AGCTCTAAAT CGGAACCCTA AAGGGAGCCC CCGATTTAGA GCTTGACGGG  
GAAAGCCGGC GAACGTGGCG AGAAAGGAAG GGAAGAAAGC GAAAGGAGCG  
GGCGCTAGGG CGCTGGCAAG TGTAGCGGTC ACGCTGCGCG TAACCACCAC  
ACCCGCCGCG CTTAATGCGC CGCTACAGGG CGCGTCCATT CGCCATTCAG  
GCTGCGCAAC TGT TGGGAAG GGCGATCGGT GCGGGCCTCT TCGCTATTAC  
GCCAGCTGGC GAAAGGGGGA TGTGCTGCAA GGCGATTAAG TTGGGTAACG  
CCAGGGTTTT CCCAGTCACG ACGTTGTAAA ACGACGGCCA GTGAATTG



FIG. 13.

## TopoRNAi

1	gagtgcacca	tatgcggtgt	gaaataccgc	acagatgcgt	aaggagaaaa	taccgcatca
61	ggcgaaattg	taaacgttaa	tattttgtta	aaattcgcgt	taaatatttg	ttaaatcagc
121	tcatttttta	accaataggc	cgaaatcggc	aaaatccctt	ataaatcaaa	agaatagacc
181	gagatagggt	tgagtgttgt	tccagtttgg	aacaagagtc	cactattaaa	gaacgtggac
241	tccaacgtca	aagggcgaaa	aaccgtctat	cagggcgatg	gcccactacg	tgaaccatca
301	cccaaataca	gttttttgcg	gtcggaggtg	cgtaaagctc	taaatcggaa	ccctaaaggg
361	agcccccgat	ttagagcttg	acggggaaag	ccggcgaaacg	tggcgagaaa	ggaagggaag
421	aaagcgaaag	gagcggggcg	tagggcgctg	gcaagtgtag	cggtcacgct	gcgcgtaacc
481	accacacccg	ccgcgcttaa	tgcgccgcta	cagggcgcgct	ccattcgcca	ttcaggctgc
541	gcaactgttg	ggaaggggcg	tccgtgcggg	cctcttcgct	attacgccag	ctggcgaaag
601	ggggatgtgc	tgcaaggcga	ttaagttggg	taacgccagg	gttttcccag	tcacgacgtt
661	gtaaaacgac	ggccagtga	ttgtaatacg	actcactata	gggcgaattc	aaaaaacccc
721	tcaagacccg	tttagaggcc	ccaaggggtt	atgctagtga	attctgcagg	gtacccgggg
781	atcctctaga	gatccctcga	cctcgagatc	cattgtgggt	gaattctacc	aaggctagca
841	tgggcagccg	aatacagtga	tccgtgccgg	ccctggactg	ttgaacgagg	tcggcgtaga
901	cggctctgacg	acacgcaaac	tggcggaacg	gttggggggtg	cagcagccgg	cgctttactg
961	gcacttcagg	aacaagcggg	cgctgctcga	cgcaactggc	gaagccatgc	tggcgagaaa
1021	tcatacgctt	cgggtgccgag	agccgacgac	gactggcgct	catttctgat	cggaatccc
1081	gcagccatgc	tagccttggt	agaattccac	cacaatggat	ctcgagggat	cttccatacc
1141	taccagttct	gcgcctgcag	gtcgcggccg	cgactctcta	gacgcgtaag	cttactagca
1201	taaccctctg	gggcctctaa	acgggtcttg	aggggttttt	tgagcttctc	gccctatagt
1261	gagtcgtatt	acagcttgag	tattctatag	tgtcacctaa	atagcttggc	gtaatcatgg
1321	tcatagctgt	ttcctgtgtg	aaattgttat	ccgctcacia	ttccacacia	catacgagcc
1381	ggaagcataa	agtgtaaaagc	ctgggggtgcc	taatgagtga	gctaactcac	attaattgcg
1441	ttgcgctcac	tgcccgcctt	ccagtcggga	aacctgtcgt	gccagctgca	ttaatgaatc
1501	ggcgaacgcg	cggggagagg	cggtttgcgt	attgggcgct	cttccgcttc	ctcgctcact
1561	gactcgctgc	gctcggctcg	tcggctgcgg	cgagcggtat	cagctcactc	aaaggcggtg
1621	atacggttat	ccacagaatc	aggggataac	gcaggaaaga	acatgtgagc	aaaaggccag
1681	caaaaggcca	ggaaccgtaa	aaaggccgcg	ttgctggcgt	ttttcgatag	gctccgcccc
1741	cctgacgagc	atcacaaaaa	tcgacgctca	agtcagaggt	ggcgaaaccc	gacaggacta
1801	taaagatacc	aggcgtttcc	ccctggaagc	tccctcgtgc	gctctcctgt	tcggaccctg
1861	ccgcttaccg	gatacctgtc	cgccctttct	ccttcgggaa	gcgtggcgct	ttctcatagc
1921	tcacgctgta	ggtatctcag	ttcgggtgtg	gtcgttcgct	ccaagctggg	ctgtgtgcac
1981	gaaccccccg	ttcagcccga	ccgctgcgcc	ttatccggta	actatcgtct	tgagtccaac
2041	ccggttaagac	acgacttatc	gccactggca	gcagccactg	gtaacaggat	tagcagagcg
2101	aggatatgtg	gcggtgctac	agagttcttg	aagtgggtgg	ctaactacgg	ctacactaga
2161	aggacagtat	ttggtatctg	cgctctgctg	aagccagtta	ccttcggaaa	aagagttggt
2221	agctcttgat	ccggcaaaca	aaccaccgct	ggtagcgggtg	gtttttttgt	ttgcaagcag
2281	cagattacgc	gcagaaaaaa	aggatctcaa	gaagatcott	tgatcttttc	tacggggtct
2341	gacgctcagt	ggaacgaaaa	ctcacgttaa	gggatttttg	tcattgagatt	atcaaaaagg
2401	atcttcacct	agatcctttt	aaattaaaaa	tgaagtttta	aatcaatcta	aagtatatat
2461	gagtaaaact	ggtctgacag	ttaccaatgc	ttaatcagtg	aggcacctat	ctcagcgatc
2521	tgtctatttc	gttcatccat	agttgcctga	ctccccgctg	tgtagataac	tacgatacgg
2581	gagggcttac	catctggccc	cagtgtgcca	atgataccgc	gagacccacg	ctcaccggct
2641	ccagatttat	cagcaataaa	ccagccagcc	ggaaggggcg	agcgagaaag	tggtccctgca
2701	actttatccg	cctccatcca	gtctattaat	tggtggccgg	aagctagagt	aagtagttcg
2761	ccagtttaata	gtttgcgcaa	cgttgttgcc	attgctacag	gcacgtgggt	gtcacgctcg
2821	tcgtttggta	tggtttcatt	cagctccggt	tcccaacgat	caaggcgagt	tacatgatcc
2881	cccatgttgt	gcaaaaaagc	ggttagctcc	ttcggctctc	cgatcgttgt	cagaagtaag
2941	ttggccgcag	tggtatcact	catgggttatg	gcagcactgc	ataattctct	tactgtcatg
3001	ccatccgtaa	gatgcttttc	tgtgactggt	gagtactcaa	ccaagtcatt	ctgagaatac
3061	cgcccccggc	gaccgagttg	ctcttgcccg	gcgtcaatac	gggataatag	tgtatgacat
3121	agcagaactt	taaaagtgtc	catcattgga	aaacgttctt	cggggcgaaa	actctcaagg
3181	atcttaccgc	tggtgagatc	cagttcgatg	taaccactc	gtgcacccaa	ctgatcttca
3241	gcattcttta	ctttcaccag	cgtttctggg	tgagcaaaaa	caggaaggca	aaatgccgca
3301	aaaaagggaa	taaggggcgac	acggaaatgt	tgaataactca	tactcttcct	ttttcaatat
3361	tattgaagca	tttatcaggg	ttattgtctc	atgagcggat	acatatttga	atgtatttag
3421	aaaaataaac	aaataggggt	tccgcgcaca	tttccccgaa	aagtgccacc	tgacgtctaa
3481	gaaaccatta	ttatcatgac	attaacctat	aaaaataggc	gtatcacgag	gccctttcgt
3541	ctcgcgcggt	tcggtgatga	cggtgaaaac	ctctgacaca	tgcagctccc	ggagacgggc
3601	acagcttgtc	tgtaagcggg	tgccgggagc	agacaagccc	gtcagggcgc	gtcagcgggt
3661	gttggcgggt	gtcgggggctg	gcttaactat	gcggcatcag	agcagattgt	actga

*PGN49A* *FIG. 14.*

TGTAATACGA CTCACTATAG GGCGAATTCA AAAAACCCTT CAAGACCCGT  
TTAGAGGCCC CAAGGGGTTA TGCTAGTGAA TTCTGCAGCG GTACCCGGGG  
ATCCTCTAGA GATCCCTCGA CCTCGAGATC CATTGTGCTG GAAAGGATCT  
GGATCCGGCT TACTAAAAGC CAGATAACAG TATGCGTATT TGC GCGCTGA  
TTTTTGGCGT ATAAGAATAT ATACTGATAT GTATACCCGA AGTATGTCAA  
AAAGAGGTGT GCTATGAAGC AGCGTATTAC AGTGACAGTT GACAGCGACA  
GCTATCAGTT GCTCAAGGCA TATATGATGT CAATATCTCC GGTCTGGTAA  
GCACAACCAT GCAGAAATGAA GCCCGTCGTC TGC GTGCCGA ACGCTGGAAA  
GCGGAAAATC AGGAAGGGAT GGCTGAGGTC GCCCGGTTTA TTGAAATGAA  
CGGCTCTTTT GCTGACGAGA ACAGGGACTG GTGAAATGCA GTTTAAGGTT  
TACACCTATA AAAGAGAGAG CCGTTATCGT CTGTTTGTGG ATGTACAGAG  
TGATATTATT GACACGCCCC GGCGACGGAT GGTGATCCCC CTGGCCAGTG  
CACGTCTCTT AAGCGATAAA GTCTCCCGTG AACTTTACCC GGTGGTGCAT  
ATCGGGGATG AAAGCTGGCG CATGATGACC ACCGATATGG CCAGTGTGCC  
GGTCTCCGTT ATCGGGGAAG AAGTGGCTGA TCTCAGCCAC CGCGAAAATG  
ACATCAAAAA CGCCATTAACT CTGATGTTCT GGGGAATATA AATGTCAGGC  
TCCCTTATAC ACAGCCTTTC CAGCACAATG GATCTCGAGG GATCTTCCAT  
ACCTACCAGT TCTGCGCCTG CAGGTCGCGG CCGCGACTCT AGACGCGTAA  
GCTTACTAGC ATAACCCCTT GGGGCTCTA AACGGGTCTT GAGGGGTTTT  
TTGAGCTTCT CGCCCTATAG TGAGTCGTAT TACAGCTTGA GTATTCTATA  
GTGTCACCTA AATAGCTTGG CGTAATCATG GTCATAGCTG TTTCTGTGT  
GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC CGGAAGCATA  
AAGTGTAAG CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAAATTGC  
GTTGCGCTCA CTGCCCCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC  
ATTAATGAAT CGGCCAACGC GCGGGGAGAG GCGGTTTTCG TATTGGGCGC  
TCTTCCGCTT CCTCGCTCAC TGACTCGCTG CGCTCGGTCG TTCGGCTGCG  
GCGAGCGGTA TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT  
CAGGGGATAA CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC  
AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCGATA GGCTCCGCCC  
CCCTGACGAG CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC  
CGACAGGACT ATAAAGATAC CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG  
CGCTCTCCTG TTCCGACCCT GCCGCTTACC GGATACCTGT CCGCCTTTCT  
CCCTTCGGGA AGCGTGGCGC TTTCTCATAG CTCACGCTGT AGGTATCTCA  
GTTCCGGTGTA GGTGCTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC  
GTTCCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA  
CCCGGTAAGA CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA  
TTAGCAGAGC GAGGTATGTA GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG  
CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT GCGCTCTGCT  
GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC  
AAACCACCGC TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG  
CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC  
TGACGCTCAG TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT  
TATCAAAAAG GATCTTCACC TAGATCCTTT TAAATTAAAA ATGAAGTTTT

*FIG. 14 (CONTINUED)*

AAATCAATCT AAAGTATATA TGAGTAAACT TGGTCTGACA GTTACCAATG  
CTTAATCAGT GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA  
TAGTTGCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA  
CCATCTGGCC CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC  
TCCAGATTTA TCAGCAATAA ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA  
GTGGTCCTGC AACTTTATCC GCCTCCATCC AGTCTATTAA TTGTTGCCGG  
GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA ACGTTGTTGG  
CATTGCTACA GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT  
TCAGCTCCGG TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG  
TGCAAAAAG CGGTTAGCTC CTTCCGTCCT CCGATCGTTG TCAGAAGTAA  
GTTGGCCGCA GTGTTATCAC TCATGGTTAT GGCAGCACTG CATAATTCTC  
TTACTGTCAT GCCATCCGTA AGATGCTTTT CTGTGACTGG TGAGTACTCA  
ACCAAGTCAT TCTGAGAATA CCGCGCCCGG CGACCGAGTT GCTCTTGCCC  
GGCGTCAATA CGGGATAATA GTGTATGACA TAGCAGAACT TTAAAAGTGC  
TCATCATTGG AAAACGTTCT TCGGGGCGAA AACTCTCAAG GATCTTACCG  
CTGTTGAGAT CCAGTTCGAT GTAACCCACT CGTGCAACCA ACTGATCTTC  
AGCATCTTTT ACTTTCACCA GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC  
AAAATGCCGC AAAAAGGGA ATAAGGGCGA CACGGAAATG TTGAATACTC  
ATACTCTTCC TTTTCAATA TTATTGAAGC ATTTATCAGG GTTATTGTCT  
CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG  
TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT  
ATTATCATGA CATTAACTA TAAAAATAGG CGTATCACGA GGCCCTTTTCG  
TCTCGCGCGT TTCGGTGATG ACGGTGAAAA CCTCTGACAC ATGCAGCTCC  
CGGAGACGGT CACAGCTTGT CTGTAAGCGG ATGCCGGGAG CAGACAAGCC  
CGTCAGGGCG CGTCAGCGGG TGTTGGCGGG TGTCGGGGCT GGCTTAACTA  
TGCGGCATCA GAGCAGATTG TACTGAGAGT GCACCATATG CGGTGTGAAA  
TACCGCACAG ATGCGTAAGG AGAAAATACC GCATCAGGCG AAATTGTAAA  
CGTTAATATT TTGTTAAAT TCGCGTTAAA TATTTGTTAA ATCAGCTCAT  
TTTTTAACCA ATAGGCCGAA ATCGGCAAAA TCCCTTATAA ATCAAAAGAA  
TAGACCGAGA TAGGGTTGAG TGTTGTTCCA GTTTGGAACA AGAGTCCACT  
ATTAAAGAAC GTGGACTCCA ACGTCAAAGG GCGAAAAACC GTCTATCAGG  
GCGATGGCCC ACTACGTGAA CCATCACCCA AATCAAGTTT TTTGCGGTCC  
AGGTGCCGTA AAGCTCTAAA TCGGAACCCT AAAGGGAGCC CCCGATTTAG  
AGCTTGACGG GGAAAGCCGG CGAACGTGGC GAGAAAGGAA GGGAAGAAAG  
CGAAAGGAGC GGGCGCTAGG GCGCTGGCAA GTGTAGCGGT CACGCTGCGC  
GTAACCACCA CACCCGCCGC GCTTAATGCG CCGCTACAGG GCGCGTCCAT  
TCGCCATTCA GGCTGCGCAA CTGTTGGGAA GGGCGATCGG TGCGGGCCTC  
TTCGCTATTA CGCCAGCTGG CGAAAGGGGG ATGTGCTGCA AGGCGATTAA  
GTTGGGTAAC GCCAGGGTTT TCCAGTCAC GACGTTGTAA AACGACGGCC  
AGTGAAT

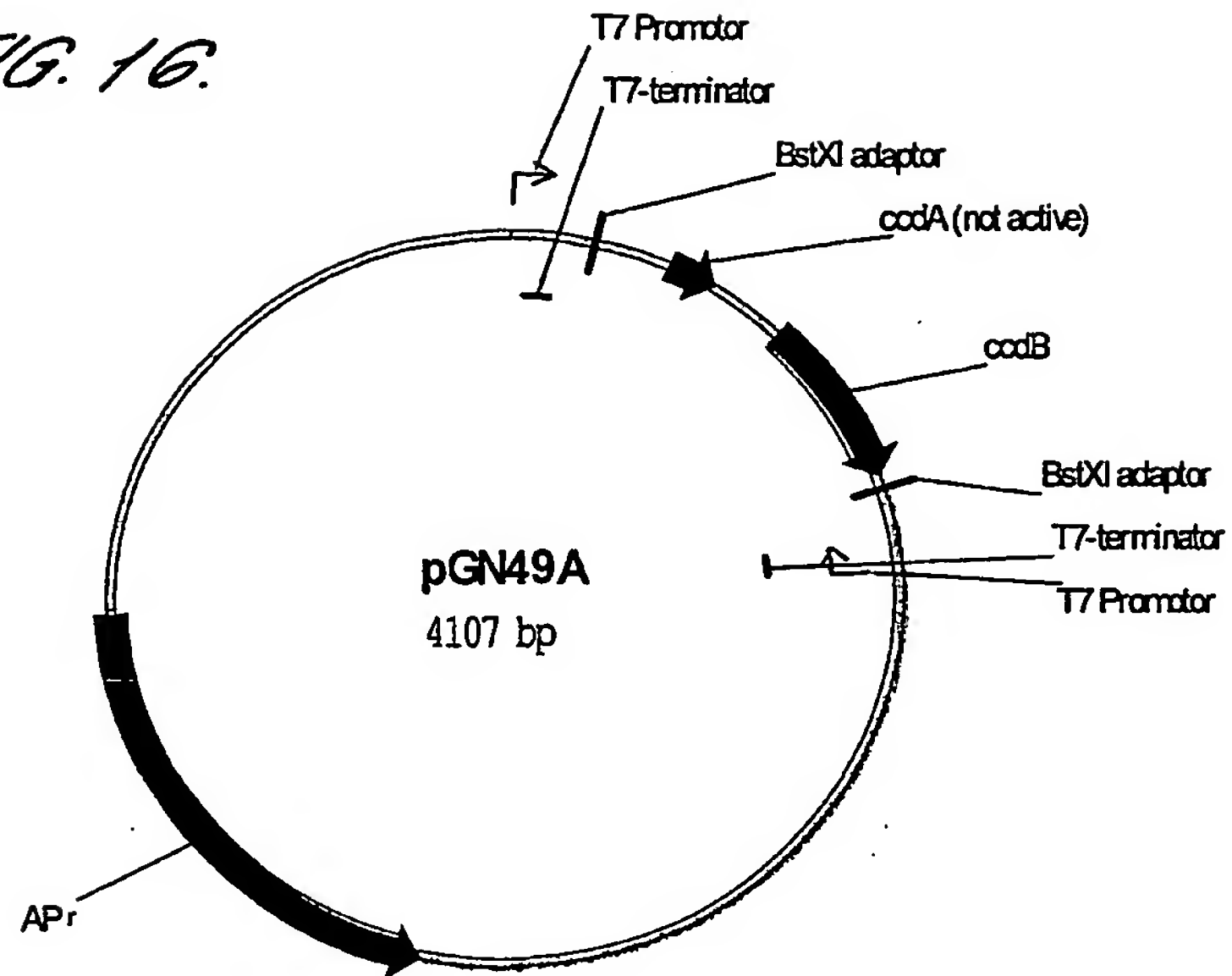
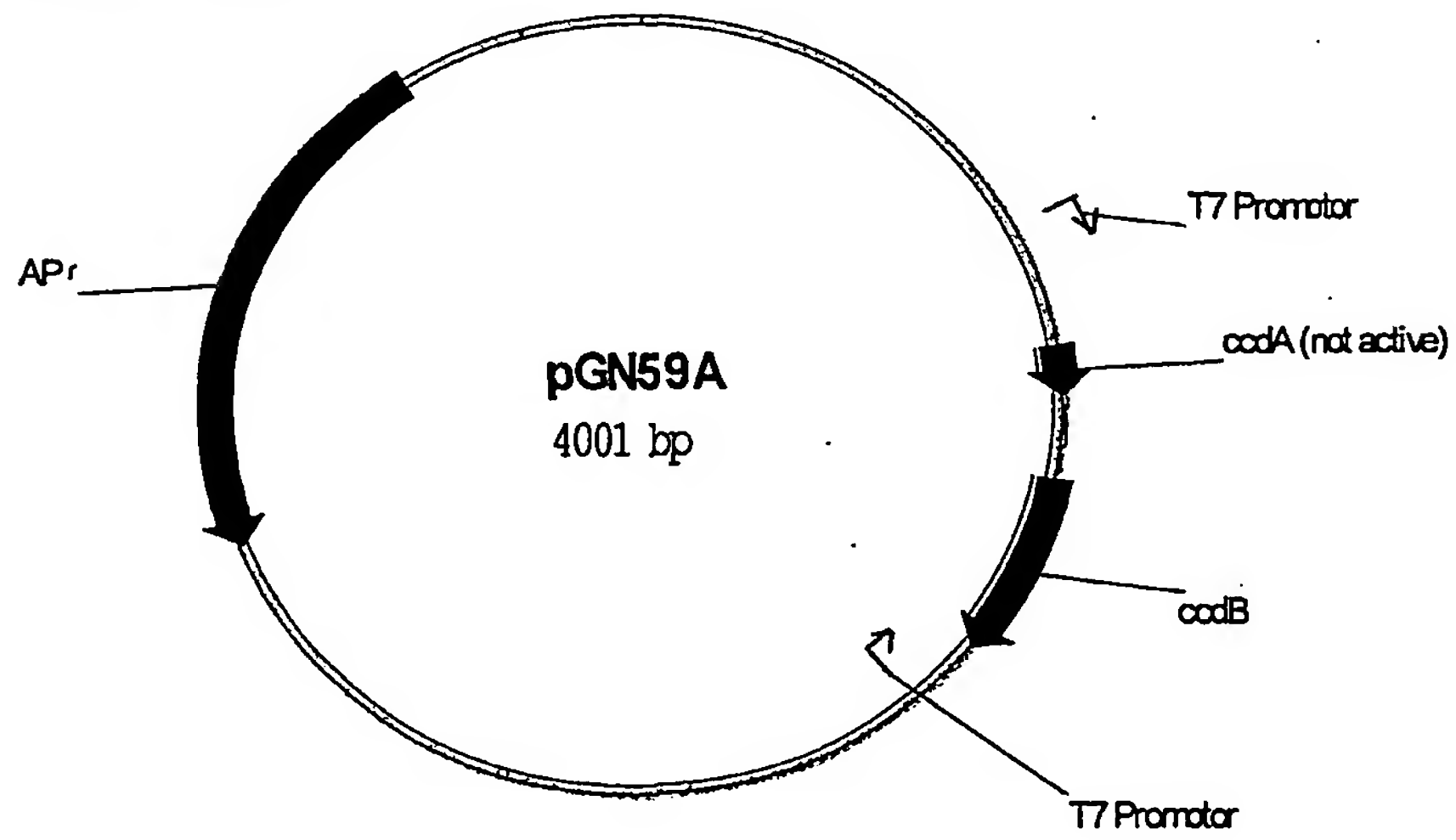


*pgn59a* *FIG. 15.*

GAGTGCACCA	TATGCGGTGT	GAAATACCGC	ACAGATGCGT	AAGGAGAAAA
TACCGCATCA	GGCGAAATTG	TAAACGTTAA	TATTTTGTTA	AAATTCGCGT
TAAATATTTG	TTAAATCAGC	TCATTTTFTA	ACCAATAGGC	CGAAATCGGC
AAAATCCCTT	ATAAATCAAA	AGAATAGACC	GAGATAGGGT	TGAGTGTTGT
TCCAGTTTGG	AACAAGAGTC	CACTATTAAA	GAACGTGGAC	TCCAACGTCA
AAGGGCGAAA	AACCGTCTAT	CAGGGCGATG	GCCCACTACG	TGAACCATCA
CCCAAATCAA	GTTTTTTGCG	GTCGAGGTGC	CGTAAAGCTC	TAAATCGGAA
CCCTAAAGGG	AGCCCCCGAT	TTAGAGCTTG	ACGGGGAAAG	CCGGCGAACG
TGGCGAGAAA	GGAAGGGAAG	AAAGCGAAAG	GAGCGGGCGC	TAGGGCGCTG
GCAAGTGTAG	CGGTCACGCT	GCGCGTAACC	ACCACACCCG	CCGCGCTTAA
TGCGCCGCTA	CAGGGCGCGT	CCATTGCGCA	TTCAGGCTGC	GCAACTGTTG
GGAAGGGCGA	TCGGTGCGGG	CCTCTTCGCT	ATTACGCCAG	CTGGCGAAAG
GGGGATGTGC	TGCAAGGCGA	TTAAGTTGGG	TAACGCCAGG	GTTTTCCCAG
TCACGACGTT	GTAAAACGAC	GGCCAGTGAA	TTGTAATACG	ACTCACTATA
GGGCGAATTC	GAGCTCGGTA	CCCGGGGATC	CTCTAGAGAT	CCCTCGACCT
CGAGATCCAT	TGTGCTGGAA	AGGATCTGGA	TCCGGCTTAC	TAAAAGCCAG
ATAACAGTAT	GCGTATTTGC	GCGCTGATTT	TTGCGGTATA	AGAATATATA
CTGATATGTA	TACCCGAAGT	ATGTCAAAAA	GAGGTGTGCT	ATGAAGCAGC
GTATTACAGT	GACAGTTGAC	AGCGACAGCT	ATCAGTTGCT	CAAGGCATAT
ATGATGTCAA	TATCTCCGGT	CTGGTAAGCA	CAACCATGCA	GAATGAAGCC
CGTCGTCTGC	GTGCCGAACG	CTGGAAAGCG	GAAAATCAGG	AAGGGATGGC
TGAGGTCGCC	CGGTTTATTG	AAATGAACGG	CTCTTTTGCT	GACGAGAACA
GGGACTGGTG	AAATGCAGTT	TAAGGTTTAC	ACCTATAAAA	GAGAGAGCCG
TTATCGTCTG	TTTGTGGATG	TACAGAGTGA	TATTATTGAC	ACGCCCCGGC
GACGGATGGT	GATCCCCCTG	GCCAGTGCAC	GTCTCTTAAG	CGATAAAGTC
TCCCGTGAAC	TTTACCCGGT	GGTGCATATC	GGGGATGAAA	GCTGGCGCAT
GATGACCACC	GATATGGCCA	GTGTGCCGGT	CTCCGTTATC	GGGGAAGAAG
TGGCTGATCT	CAGCCACCGC	GAAAATGACA	TCAAAAACGC	CATTAACCTG
ATGTTCTGGG	GAATATAAAT	GTCAGGCTCC	CTTATACACA	GCCTTTCCAG
CACAATGGAT	CTCGAGGGAT	CTTCATACC	TACCAGTTCT	GCGCCTGCAG
GTCGCGGCCG	CGACTCTCTA	GAGTCGAAAG	CTTCTCGCCC	TATAGTGAGT
CGTATTACAG	CTTGAGTATT	CTATAGTGTC	ACCTAAATAG	CTTGGCGTAA
TCATGGTCAT	AGCTGTTTCC	TGTGTGAAAT	TGTTATCCGC	TCACAATTCC
ACACAACATA	CGAGCCGGAA	GCATAAAGTG	TAAAGCCTGG	GGTGCCTAAT
GAGTGAGCTA	ACTCACATTA	ATTGCGTTGC	GCTCACTGCC	CGCTTTCCAG
TCGGGAAACC	TGTCGTGCCA	GCTGCATTAA	TGAATCGGCC	AACGCGCGGG
GAGAGGCGGT	TTGCGTATTG	GGCGCTCTTC	CGCTTCCTCG	CTCACTGACT
CGCTGCGCTC	GGTCGTTCCG	CTGCGGCGAG	CGGTATCAGC	TCACTCAAAG
GCGGTAAATAC	GGTTATCCAC	AGAATCAGGG	GATAACGCAG	GAAAGAACAT
GTGAGCAAAA	GGCCAGCAAA	AGGCCAGGAA	CCGTAAAAAG	GCCGCGTTGC
TGGCGTTTTT	CGATAGGCTC	CGCCCCCTG	ACGAGCATCA	CAAAAATCGA
CGCTCAAGTC	AGAGGTGGCG	AAACCCGACA	GGACTATAAA	GATACCAGGC
GTTTCCCCCT	GGAAGCTCCC	TCGTGCGCTC	TCCGTGTTCCG	ACCCTGCCGC
TTACCGGATA	CCTGTCCGCC	TTTCTCCCTT	CGGGAAGCGT	GCGCTTTCT

*FIG. 15 (CONTINUED)*

CATAGCTCAC GCTGTAGGTA TCTCAGTTCG GTGTAGGTCG TTCGCTCCAA  
GCTGGGCTGT GTGCACGAAC CCCCCGTTCA GCCCGACCGC TGCGCCTTAT  
CCGGTAACTA TCGTCTTGAG TCCAACCCGG TAAGACACGA CTTATCGCCA  
CTGGCAGCAG CCACTGGTAA CAGGATTAGC AGAGCGAGGT ATGTAGGCGG  
TGCTACAGAG TTCTTGAAGT GGTGGCCTAA CTACGGCTAC ACTAGAAGGA  
CAGTATTTGG TATCTGCGCT CTGCTGAAGC CAGTTACCTT CGGAAAAAGA  
GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA GCGGTGGTTT  
TTTTGTTTGC AAGCAGCAGA TTACGCGCAG AAAAAAGGA TCTCAAGAAG  
ATCCTTTGAT CTTTTCTACG GGGTCTGACG CTCAGTGGAA CGAAAACTCA  
CGTTAAGGGA TTTTGGTCAT GAGATTATCA AAAAGGATCT TCACCTAGAT  
CCTTTTAAAT TAAAAATGAA GTTTTAAATC AATCTAAAGT ATATATGAGT  
AAACTTGGTC TGACAGTTAC CAATGCTTAA TCAGTGAGGC ACCTATCTCA  
GCGATCTGTC TATTTCTGTC ATCCATAGTT GCCTGACTCC CCGTCGTGTA  
GATAACTACG ATACGGGAGG GCTTACCATC TGGCCCCAGT GCTGCAATGA  
TACCGCGAGA CCCACGCTCA CCGGCTCCAG ATTTATCAGC AATAAACCAG  
CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT CCTGCAACTT TATCCGCCTC  
CATCCAGTCT ATTAATTGTT GCCGGGAAGC TAGAGTAAGT AGTTCGCCAG  
TTAATAGTTT GCGCAACGTT GTTGGCATTG CTACAGGCAT CGTGGTGTCA  
CGCTCGTCGT TTGGTATGGC TTCATTACAGC TCCGGTTCCC AACGATCAAG  
GCGAGTTACA TGATCCCCCA TGTTGTGCAA AAAAGCGGTT AGCTCCTTCG  
GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG CCGCAGTGTT ATCACTCATG  
GTTATGGCAG CACTGCATAA TTCTCTTACT GTCATGCCAT CCGTAAGATG  
CTTTTCTGTG ACTGGTGAGT ACTCAACCAA GTCATTCTGA GAATACCGCG  
CCCGGCGACC GAGTTGCTCT TGCCCGGCGT CAATACGGGA TAATAGTGTA  
TGACATAGCA GAACTTTAAA AGTGCTCATC ATTGGAAAAC GTTCTTCGGG  
GCGAAAACCTC TCAAGGATCT TACCGCTGTT GAGATCCAGT TCGATGTAAC  
CCACTCGTGC ACCCAACTGA TCTTCAGCAT CTTTACTTT CACCAGCGTT  
TCTGGGTGAG CAAAAACAGG AAGGCAAAAT GCCGCAAAAA AGGGAATAAG  
GGCGACACGG AAATGTTGAA TACTCATACT CTTCCTTTTT CAATATTATT  
GAAGCATTTA TCAGGGTTAT TGTCTCATGA GCGGATACAT ATTTGAATGT  
ATTTAGAAAA ATAAACAAAT AGGGGTTCCG CGCACATTTT CCCGAAAAGT  
GCCACCTGAC GTCTAAGAAA CCATTATTAT CATGACATTA ACCTATAAAA  
ATAGGCGTAT CACGAGGCCC TTTCGTCTCG CGCGTTTCGG TGATGACGGT  
GAAAACCTCT GACACATGCA GCTCCCGGAG ACGGTCACAG CTTGTCTGTA  
AGCGGATGCC GGGAGCAGAC AAGCCCGTCA GGGCGCGTCA GCGGGTGTG  
GCGGGTGTCT GGGCTGGCTT AACTATGCGG CATCAGAGCA GATTGTACTG  
A

*FIG. 16.**FIG. 17.*

## SEQUENCE LISTING

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<130> SCB/55178/001

<140>

<141>

<160> 21

<170> PatentIn Ver. 2.0

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<211> 160

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Fragment of  
pGN1 containing opposable T7 promoters

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gaaagcttct cgccctatag tgagtcgtat tacagcttga gtattctata gtgtcaccta 120  
aatagcttgg cgtaatcatg gtcatagctg tttcctgtgt 160

<210> 2

<211> 49

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: DNA sequence  
containing a T7 terminator

<400> 2

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<210> 3

<211> 70

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:  
Oligonucleotide oGN27

<400> 3

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gcagcggtac 70

<210> 4

<211> 62

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:

## Oligonucleotide oGN28

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tg 62

&lt;210&gt; 5

&lt;211&gt; 65

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:  
Oligonucleotide oGN29

&lt;400&gt; 5

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ttttg 65

&lt;210&gt; 6

&lt;211&gt; 65

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence:  
Oligonucleotide oGN30

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cgcg 65

&lt;210&gt; 7

&lt;211&gt; 230

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Fragment of  
plasmid pGN9 containing opposable T7 promoters and  
T7 transcription terminators

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ccaaggggtt atgctagtga attctgcagg gtaccgggg atcctctaga cgcgtaagct 120  
tactagcata accccttggg gcctctaaac gggctctgag gggttttttg agcttctcgc 180  
cctatagtga gtcgtattac agcttgagta ttctatagtgc tcacctaaat 230

&lt;210&gt; 8

&lt;211&gt; 3323

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Plasmid pGN9

&lt;400&gt; 8

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tcatttttta accaataggc cgaaatcggc aaaatccctt ataaatcaaa agaataagacc 180  
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ggcatcagag cagattgtac tga 3323

&lt;210&gt; 9

&lt;211&gt; 3774

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Plasmid pGN29



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<210> 10

<211> 5148

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGN39

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&lt;211&gt; 3715

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Plasmid  
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<211> 4107

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGN49A

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<211> 4001

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Plasmid pGN59A

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gagttgctct tgcccggcgt caatacggga taatagtgtg tgacatagca gaactttaaa 3420  
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gagatccagt tcgatgtaac ccactcgtgc acccaactga tcttcagcat cttttacttt 3540  
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ggcgacacgg aaatgttgaa tactcatact cttcctttt caatattatt gaagcattta 3660  
tcagggttat tgtctcatga gcggatacat atttgaatgt atttagaaaa ataaacaaat 3720  
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tgatgacggt gaaaacctct gacacatgca gtcgccggag acggtcacag cttgtctgta 3900  
agcggatgcc gggagcagac aagcccgctc gggcgcgctc gcgggtgttg gcgggtgtcg 3960  
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<210> 14

<211> 36

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<213> Artificial Sequence

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<223> Description of Artificial Sequence:  
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<212> DNA

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<223> Description of Artificial Sequence:  
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ccctggactg ttgaacgagg tcggcgtaga cggctctgacg acacgcaaac tggcgggaacg 180  
gttgggggtg cagcagccgg cgctttactg gcacttcagg aacaagcggg cgctgctcga 240  
cgcactggcc gaagccatgc tggcggagaa tcatacgctt cggtgccgag agccgacgac 300  
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gtaccagcac aatgg 375

<210> 21  
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<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR fragment



&lt;400&gt; 21

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gaagcagcgt	attacagtga	cagttgacag	cgacagctat	cagttgctca	aggcatatat	180
gatgtcaata	tctccggtct	ggtaagcaca	accatgcaga	atgaagccc	tcgtctgcgt	240
gccgaacgct	ggaaagcgga	aaatcaggaa	gggatggctg	aggtcgccc	gtttattgaa	300
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ctataaaaga	gagagccggt	atcgtctggt	tgtggatgta	cagagtgata	ttattgacac	420
gcccgggcga	cggatgggta	tccccctggc	cagtgcacgt	ctcttaagcg	ataaagtctc	480
ccgtgaactt	tacccggtgg	tgcatatcgg	ggatgaaagc	tggcgcatga	tgaccaccga	540
tatggccagt	gtgccggtct	cgttatcgg	ggaagaagtg	gctgatctca	gccaccgcga	600
aatgacatc	aaaaacgcca	ttaacctgat	gttctgggga	atataaatgt	caggctccct	660
tatacacagc						670

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IB 01/01068A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12N15/10 C12N15/63 C12N15/70 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FR 2 782 325 A (PROTEUS) 18 February 2000 (2000-02-18) page 7, line 20 -page 8, line 8 page 11, line 11 - line 36 page 23, line 31 -page 24, line 9 ---	1-24, 26, 27
A	WO 00 01846 A (DEVGEN N.V.) 13 January 2000 (2000-01-13) cited in the application page 8, line 9 -page 10, line 22 page 15, line 9 - line 33 page 21, line 21 -page 22, line 29 --- -/--	1-28

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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- \*P\* document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search

20 September 2001

Date of mailing of the international search report

27/09/2001

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Montero Lopez, B

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IB 01/01068

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>WO 01 34815 A (CAMBRIA BIOSCIENCES, LLC)  17 May 2001 (2001-05-17)  page 5, last paragraph -page 6, paragraph 4  page 20, paragraph 2  page 24, last paragraph; example 1  page 13, last paragraph -page 15, paragraph 2</p> <p>-----</p>	<p>1-10, 12,  22, 23, 25</p>

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Information on patent family members

ational Application No

PCT/IB 01/01068

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			AU 5171699 A	06-03-2000
			BR 9912934 A	08-05-2001
			EP 1104489 A1	06-06-2001
			WO 0009747 A1	24-02-2000
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			WO 0001846 A2	13-01-2000
			EP 1093526 A2	25-04-2001
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			NO 20010019 A	05-03-2001
WO 0134815	A	17-05-2001	AU 1461701 A	06-06-2001
			WO 0134815 A1	17-05-2001